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http://actabiomedica.ru E-mail: journalirk@gmail.com

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EDITOR-IN-CHIEF'S PREFACE

EDITOR-IN-CHIEF'S PREFACE TO ISSUE 6, 2023

Sergey I. Kolesnikov

Member of the RAS

We begin the review of the issue with the still relevant topics – **COVID-19** and other infections, since they not only do not leave us alone, but are leading the list of frontiers and superfrontiers of science in 2023. Everyone hopes for specific prevention, so we would like to mention an article by an international team from Spain and Irkutsk (Aaromal Ajitha Sureshkumar et. al.) on the attitudes assessment towards children vaccination among parents in India. Most of the parents support their children's vaccinating, but they care little about the possible problems associated with it.

Another direction is the nonspecific **prevention of respiratory viral infections**, which is the subject of the article by A.A. Ruleva et al. (Saint Petersburg) on the positive prospects for preventing with "Thymogen spray", which was used in young people for 10 days, and contributed to an increase in the production of α-interferon in response to the viral pathogen *in vitro*.

An important direction in the diagnosis and prognosis of **COVID-19** course is the assessment of the **role of genetic factors**, which is the subject of the article by M.V. Osikov et al. (Chelyabinsk), who revealed a change in the frequencies of certain *ITGB3*, *GP1B1* and *ITGA2* genes polymorphisms occurrence (with the exception of the rs6065 polymorphism) in platelet hyperreactivity in **COVID-19-associated** moderate and severe lung lesions. This subject is also covered in the article by the team of the Member of the RAS D.A. Sychev (Abdullaev Sh.P. et al.), who did not found association of rs11385942 and rs657152 variants with the severity of the course and outcomes of COVID-19 in patients treated with favipiravir and remdesivir.

More and more attention is paid **to the consequences of COVID-19**. Study by I.A. Cherevikova et al. (Irkutsk) revealed increased asthenic conditions, attention disorders, high anxiety, and severe depression in adolescents with post-COVID syndrome, especially in the second month after COVID-19.

Another respiratory infection, **influenza**, is the subject of the article by E.D. Kazantseva et al. (Irkutsk), who determined a significant increase in the level of pro-inflammatory cytokines IL1β, IL6, IL8, TNF-α, as well as C-reactive protein, anti-inflammatory cytokine IL4 in all age categories of adolescents with influenza, but no gender differences were revealed.

One virologist said figuratively, "The clock of the new pandemic is ticking, we just don't know what time it is." And the Director-General of WHO warned that a new pandemic is quite possible. Therefore, the identification of natural **potentially dangerous foci of infections** is a very urgent problem. There are **potentially dangerous coronaviruses** circulating not only in bats, but also in rodents and insectivores, and that was shown in the work of L.N. Yashina et al. (Novosibirsk). The authors found five species of natural carriers of three different coronaviruses on the territory of the Altai Republic, and the virus identified from the insectivorous probably belongs to the new subgenus *Coronaviridae*. It is somewhat reassuring that the authors revealed a relatively low rate of evolution of these viruses.

As a result of human economic activity, the habitat of vectors of tick-borne **infections** will melt, especially in poorly studied areas of Siberia. The article by E.K. Lagunova et al. (Irkutsk) gives a detailed description of such infections in the buffer zone of the Baikal natural territory. A conclusion is made about the wide distribution of active natural foci of tick-borne encephalitis, Lyme disease, tick-borne relapsing fever caused by *B. miyamotoi*, granulocytic anaplasmosis, and human monocytic ehrlichiosis. This topic is complemented by the work of V.A. Rar et al. (from several institutes of Novosibirsk, Omsk

and Irkutsk) on the first time identified genetic heterogeneity of the population of *R. helvetica* in *Ixodes* spp., collected in Siberia and the Far East.

The topic of infections dangerous to humans is also discussed in the study of L.A. Stepanenko et al. (Irkutsk, Novosibirsk), in which using bioinformatics analysis, data were obtained about the **CRISPR system** of ***Klebsiella pneumoniae*** strains, the action of which is aimed against bacteriophages, which can contribute to the development of personalized phage therapy.

Our country is one of the most affected by **tuberculosis infection**, and it is important to predict drug resistance to new medicinse based on molecular biological data. This is the subject of an article by V.V. Sinkov et al. (Irkutsk), who in predicting resistance to bedaquiline successfully tested a system of automated interpretation of results using three strains of bacteria obtained in Yakutia.

One of the central problems is **obesity as a comorbid component** of noninfectious pathology. A team lead by member of RAS O.L. Barbarash from Kemerovo (Tsygankova D.P. et al.) presented the three-year follow-up of a large contingent of patient's data. According to most indicators of obesity (except visceral obesity index), new cases of obesity developed in 30.6 % of the examined subjects. Consequently, individual indices are not able to fully reflect the gender and age characteristics of the distribution of fat in the body. Another problem is obesity prevention, and it is very important to assess the actual nutrition of adolescents. This topic is especially poorly studied in rural schoolchildren – they were the subject of the article of L.V. Rychkova et al. (Irkutsk), who revealed insufficient energy value of the diet, deficiency of proteins and fats, macro- and micronutrients (vitamins A, C and D), essential microelements, dietary fiber, but increased sodium intake.

An important topic of **adolescent health** is also explored in the article by M.S. Nerovnykh (Khakassia). It has been revealed that the financial well-being of the family, both directly and indirectly, can contribute to the variability of the indicators of the higher mental functions of children, which is manifested in the choice of the leading strategy for information processing.

Sleep disorders are another recognized risk factor for various non-communicable and even infectious diseases. Bolshakova S.E. et al. (Irkutsk) showed that 40.52 % of the surveyed 422 adolescent girls in Irkutsk had sleep problems: increased sleep latency, later bedtime, earlier awakening, reduced sleep time, as well as an increase in sleep shift. Another article by the authors from Institute of Medical and Biological Problems and Sechenov University (Kovrov G.V. et al.) describes the dynamics of subjective changes in the assessment of sleep quality in people under conditions of three-week antiorthostatic hypokinesia (a model of weightlessness in space flight). It was revealed that the most negative changes were observed in the first three days, with a further increase in daytime sleepiness.

Since sleep disorders are currently associated with the accumulation of amyloid proteins and the risk of developing **neurodegenerative diseases**, a review by V.N. Salkov and D.N. Voronkov (Moscow) is of interest. The authors concluded that conformationally altered alpha-synuclein can affect neurons through interaction with neuroglial cells, as well as modulate the aggregation and expression of proteins significant for the development of neurodegeneration.

Traditionally, an interesting work was presented by a team of authors under the guidance of D.A. Sychev (Moscow) on the **interdrug interaction** of Rivaroxaban and Verapamil in patients over 80 years of age with atrial fibrillation, where to avoid complications in patients taking rivaroxaban it is recommended to study the genotype of *ABCB1* (rs 4148738 and rs4148738) before adding to therapy a P-gp inhibitor. Another article on cardiovascular pathology was presented by a team from Tomsk (Chumakova S.P. et al.). The authors revealed that in case of **ischemic cardiomyopathy**, the maturation of vessels in the myocardium is impaired, but there is no activation of cellular and humoral factors of angiogenesis.

Our journal periodically publishes descriptions of unique clinical cases. This issue includes the article by M.V. Sinitsyn and N.A. Pozdeeva (Cheboksary) on the advantage of combined method of regular high degree postkeratoplas-

tic astigmatism correction with implantation of intrastromal corneal segments in a patient with cataract.

I would like to conclude my preface with an analysis of the **experimental papers** submitted to the journal. Important for the understanding and treatment of pain syndrome is the work of our colleagues from the Republic of Belarus (Erofeeva A.-M.V. et al.), who, in a model of peripheral neuropathy in Wistar male rats, showed that blockade of cannabinoid CB2-receptors both on the membranes of the injected mesenchymal stem cells and in the area of peripheral nerve injury is accompanied by a decrease in the antinociceptive effect of MSCs and suppresses their reparative effect. In the interesting work by M.A. Dymova et al. (Novosibirsk), the **cytotoxic effect of the recombinant VV-GMCSF-Lact virus** on 3D cultures of human U-87 MG glioblastoma cells was shown, which opens opportunities for the development of oncolytic therapy. Work by S.Yu. Batueva et al. (Ulan-Ude) is dedicated to the search for herbal medicines. The authors compared different methods for experimental **paracetamol hepatitis therapy**, and proved the prospects for sea buckthorn leaves extract in combination with ademethionine.

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ПРЕДИСЛОВИЕ ГЛАВНОГО РЕДАКТОРА К № 6 (2023)

Колесников Сергей Иванович

Академик РАН

Начнём с всё ещё актуальной темы – **COVID-19** – и тем, связанных с инфекциями, поскольку они не только не оставляют нас в покое, но и являются ведущими в списке фронтиров и суперфронтиров науки в 2023 году. Поскольку большинство уповают на специфическую профилактику, отметим статью международного коллектива из Испании и Иркутска (Aaromal Ajitha Sureshkumar и соавт.), оценивших отношение родителей из Индии к вакцинации своих детей. Они поддерживают вакцинацию, но их мало заботят связанные с ней возможные проблемы.

Важным направлением является **иммунопрофилактика респираторных вирусных инфекций**, которой посвящена статья А.А. Рулевой и соавт. (Санкт-Петербург), рассматривающая перспективность использования для этого «Тимоген спрея», который при применении у молодых людей в течение 10 дней способствовал увеличению продукции α-интерферона в ответ на индуцирующее воздействие вирусного патогена.

Важным направлением в оценке и прогнозе течения **COVID-19** является **оценка роли генетических факторов**, чему посвящена статья М.В. Осикова и соавт. (Челябинск), выявивших изменение частоты встречаемости отдельных полиморфизмов генов *ITGB3*, *GP1B1* и *ITGA2* (за исключением полиморфизма rs6065) при гиперреактивности тромбоцитов при COVID-19-ассоциированном поражении лёгких средней и тяжёлой степени, а также статья коллектива под руководством академика РАН Д.А. Сычева (Абдуллаев Ш.П. и соавт.), в которой не выявлена ассоциация вариантов rs11385942 и rs657152 с тяжестью течения и исходами COVID-19 у пациентов, получавших терапию фавипиравиром и ремдесивиром.

Растёт внимание к **последствиям COVID-19**, чему посвящено исследование И.А. Черевиковой и соавт. (Иркутск) по увеличению у подростков с постковидным синдромом частоты астенических состояний, нарушений внимания, высокой тревожности и выраженной депрессии, особенно на второй месяц после COVID-19.

К сожалению, нас не покидают и такие респираторные инфекции, как грипп, который исследуется Е.Д. Казанцевой и соавт. (Иркутск), показавшими статистически значимое повышение уровня провоспалительных цитокинов IL-1β, IL-6, IL-8, TNF-α, INF-α, а также С-реактивного белка, противовоспалительного цитокина IL-4 при гриппе во всех возрастных категориях подростков, но не выявивших гендерных различий.

Один вирусолог образно сказал: «часы новой пандемии тикают, просто мы не знаем, который час». Генеральный директор ВОЗ также предупредил, что новая пандемия вполне вероятна. Поэтому выявление природных **потенциально опасных очагов инфекций** – очень актуальная проблема. Есть и **потенциально опасные коронавирусы**, циркулирующие не только у рукокрылых, но и у грызунов и насекомоядных (работа Л.Н. Яшиной и соавт. (Новосибирск)). Авторы обнаружили 5 видов природных носителей 3 различных коронавирусов на территории Республики Алтай, причём выявленный от насекомоядного вирус, вероятно, относится к новому подроду *Coronaviridae*. Несколько успокаивает, что авторы выявили относительно низкую скорость эволюции этих вирусов.

Вследствие хозяйственной деятельности человека растёт ареал обитания переносчиков **клещевых инфекций**, особенно в малоизученных районах Сибири. В статье Е.К. Лагуновой и соавт. (Иркутск) дана подробна характеристика таких инфекций в буферной зоне Байкальской природной территории. Сделан вывод о широком распространении в долине р. Чикой

активных природных очагов клещевого энцефалита, болезни Лайма, клещевой возвратной лихорадки, вызываемой *B. miyamotoi*, гранулоцитарного анаплазмоза и моноцитарного эрлихиоза человека. Дополняет эту тему работа В.А. Рар и соавт. (Новосибирск, Омск, Иркутск), впервые выявивших генетическую гетерогенность популяции *R. helvetica* в *Ixodes* spp., собранных в Сибири и на Дальнем Востоке.

Тему опасных для человека инфекций продолжает исследование Л.А. Степаненко и соавт. (Иркутск, Новосибирск), где с помощью биоинформационного анализа у **штаммов *Klebsiella pneumoniae*** выявлены **CRISPR-системы**, действие которых нацелено против бактериофагов, что может содействовать разработке персонализированной фаготерапии.

Наша страна относится к числу наиболее поражённых туберкулёзом, и актуальным является прогнозирование по молекулярно-биологическим данным его лекарственной устойчивости к новым препаратам. В статье В.В. Синькова и соавт (Иркутск) при прогнозировании устойчивости к бедаквилину на трёх штаммах бактерий из Якутии успешно апробирована система автоматизированной интерпретации результатов.

Одной из центральных проблем является **ожирение** как коморбидный компонент неинфекционной патологии. Коллективом под руководством академика О.Л. Барбараш (Цыганкова Д.П. и соавт., Кемерово) представлены данные трёхлетнего наблюдения большого контингента пациентов. У 30,6 % обследованных развились новые случаи ожирения по большинству показателей (кроме важного индекса висцерального ожирения). Следовательно, отдельные индексы ожирения не способны в полной мере отразить половозрастные особенности распределения жира в организме, и необходимо использовать комплекс показателей. Продолжает тему профилактики ожирения оценка фактического питания подростков, которое слабо изучено у сельских школьников, чему и посвящена статья Л.В. Рычковой и соавт. (Иркутск), выявивших недостаточную энергетическую ценность рациона, дефицит белков и жиров, макро- и микронутриентов – витаминов A, C и D, эссенциальных микроэлементов, пищевых волокон, но повышенное потребление натрия.

Тема **здоровья подростков** исследуется и в статье М.С. Неровных (Хакассия). Выявлено, что материальный достаток семьи как непосредственно, так и опосредованно может вносить вклад в изменчивость показателей высших психических функций детей, что проявляется в выборе ведущей стратегии обработки информации.

Нарушения сна – признанный фактор риска различных неинфекционных и даже инфекционных заболеваний. С.Е. Большаковой и соавт. (Иркутск) показано, что у 40,52 % из 422 опрошенных девочек-подростков города Иркутска есть проблемы со сном, что свидетельствует о важности их выявления. А вторая статья авторов из ГНЦ РФ – ИМБП РАН и Сеченовского университета (Ковров Г.В. и соавт.) описывает динамику субъективных изменений оценки качества сна у людей в условиях трёхнедельной антиортостатической гипокинезии (модель невесомости в космическом полете). Выявлено, что наиболее негативные изменения отмечались в первые 3 дня с увеличением в дальнейшем дневной сонливости.

Поскольку в настоящее время нарушения сна связываются с накоплением амилоидных белков и риском развития **нейродегенеративных заболеваний**, представляет интерес обзор В.Н. Салькова и Д.Н. Воронкова (Москва), пришедших к выводу о том, что конформационно изменённый альфа-синуклеин может влиять на нейроны через взаимодействие с клетками нейроглии, а также модулировать агрегацию и экспрессию значимых для развития нейродегенерации белков.

Традиционно интересная работа представлена коллективом авторов под руководством Д.А. Сычева (Москва) по **межлекарственному взаимодействию** ривароксабана и верапамила у пациентов старше 80 лет с фибрилляцией предсердий, где принимающим ривароксабан пациентам во избежание осложнений рекомендуется исследование генотипа *ABCB1* (rs4148738

и rs4148738) перед добавлением к терапии ингибитора P-gp. Ещё одна статья по сердечно-сосудистой патологии представлена коллективом из Томска (Чумакова С.П. и соавт.). Авторы выявили, что при **ишемической кардиомиопатии** нарушается созревание сосудов в миокарде, но отсутствует реакция активации клеточных и гуморальных факторов ангиогенеза.

Мы иногда публикуем описание полезных для клиницистов клинических случаев – как, например, в статье М.В. Синицына и Н.А. Поздеевой (Чебоксары) о преимуществе коррекции при катаракте регулярного посткератопластического астигматизма высокой степени с использованием имплантации интрастромальных роговичных сегментов.

Завершить своё предисловие я бы хотел анализом **экспериментальных работ**, поступивших в журнал. Важна для понимания и лечения болевого синдрома работа наших коллег из Республики Беларусь (Ерофеева А.-М.В. и соавт.), которые на модели периферической нейропатии у крыс-самцов Wistar показали, что **блокада каннабиноидных CB_2-рецепторов** как на мембранах введённых мезенхимальных стволовых клеток, так и в зоне повреждения периферического нерва сопровождается снижением антиноцицептивного эффекта МСК и подавляет их репаративное действие. В интересной работе М.А. Дымовой и др. (Новосибирск) показано **цитотоксическое действие рекомбинантного вируса VV-GMCSF-Lact** на 3D-культуры клеток глиобластомы человека U-87 MG, что открывает возможности для разработки онколитической терапии. Работа С.Ю. Батуевой и соавт. (Улан-Удэ) посвящена поиску растительных лекарственных препаратов. При сравнении разных способов **терапии парацетамолового гепатита** в эксперименте показана перспективность применения экстракта листьев облепихи крушиновидной в сочетании с адеметионином.

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СОДЕРЖАНИЕ

CONTENTS

INTERNAL DISEASES

ROLE OF *ITGB3*, *GP1B1*, AND *ITGA2* GENE POLYMORPHISMS IN PLATELET DYSFUNCTION IN PATIENTS WITH COVID-19-ASSOCIATED LUNG DAMAGE

Osikov M.V. [1, 2],
Antonov V.N. [1, 3],
Zotov S.O. [1, 3]

[1] South Ural State Medical University (Vorovskogo str. 64, Chelyabinsk 454048, Russian Federation)
[2] Chelyabinsk Regional Clinical Hospital (Vorovskogo str. 70, Chelyabinsk 454048, Russian Federation)
[3] Regional Clinical Hospital No. 3 (Pobedy Ave. 271, Chelyabinsk 454136, Russian Federation)

Corresponding author:
Semen O. Zotov,
e-mail: semenz2007@yandex.ru

ABSTRACT

The aim of the work. *To investigate platelet aggregation, polymorphism in the genes that ensure its implementation, and the association between these indicators in patients with COVID-19-associated lung damage, depending on the severity of the clinical course.*

Methodology. *The study involved 75 patients with COVID-19, which, depending on the severity of lung involvement, were divided into two groups: patients with damage of up to 50 % of the lung parenchyma (n = 48) and with damage of more than 50 % (n = 27), respectively. The control group consisted of healthy people (n = 24), comparable in gender and age. In all individuals, the number of platelets, platelet aggregation induced by ADP, collagen and ristomycin were studied; polymorphisms rs6065 in the GP1BA gene, rs1126643 in the ITGA2 gene, and rs5918 in the ITGB3 gene were determined by polymerase chain reaction. Analysis of the data obtained was executed using the IBM SPSS Statistics v. 23 (IMB Corp., USA).*

Results and discussion. *In patients with moderate and severe COVID-19-associated lung damage, platelet aggregation induced by ADP, collagen, and ristomycin accelerated; in severe cases, the number of platelets decreased. The frequency of variants of the rs6065 polymorphism did not change, the frequency of occurrence of the T/C genotype of the rs5918 polymorphism increased; with moderate severity, the frequency of occurrence of the C/T and T/T genotypes of the rs1126643 polymorphism increased; with severe lung damage, the frequency of occurrence of the mutant C/C genotype polymorphism rs5918 increased. In moderate lung damage, the presence of the mutant T/T polymorphism rs1126643 accelerated collagen-induced platelet aggregation; in severe cases, the presence of mutant C/C and heterozygous variant C/T polymorphism rs5918 accelerated ADP-induced platelet aggregation. There was no effect of the rs6065 polymorphism on platelet aggregation. The data obtained indicate the possible role of genetic predisposition in the activation of platelet aggregation in patients with COVID-19-associated lung damage.*

Key words: *COVID-19, platelets, aggregation, polymorphism, GP1BA, ITGA2, ITGB3*

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РОЛЬ ПОЛИМОРФИЗМА ГЕНОВ *ITGB3*, *GP1B1* И *ITGA2* В ПАТОГЕНЕЗЕ ГИПЕРРЕАКТИВНОСТИ ТРОМБОЦИТОВ ПРИ COVID-19-АССОЦИИРОВАННОМ ПОРАЖЕНИИ ЛЁГКИХ СРЕДНЕЙ И ТЯЖЁЛОЙ СТЕПЕНИ ТЯЖЕСТИ

Осиков М.В. [1, 2],
Антонов В.Н. [1, 3],
Зотов С.О. [1, 3]

[1] ФГБОУ ВО «Южно-Уральский государственный медицинский университет» Минздрава России (454048, г. Челябинск, ул. Воровского, 64, Россия)
[2] ГБУЗ «Челябинская областная клиническая больница» (454048, г. Челябинск, ул. Воровского, 70, Россия)
[3] ГАУЗ «Областная клиническая больница № 3» (454136, г. Челябинск, просп. Победы, 287, Россия)

Автор, ответственный за переписку:
Зотов Семён Олегович,
e-mail: semenz2007@yandex.ru

РЕЗЮМЕ

Цель работы. *Исследовать агрегацию тромбоцитов, полиморфизм в генах, обеспечивающих её реализацию, и ассоциацию между данными показателями у пациентов с COVID-19 при среднем и тяжёлом течении заболевания.*

Методология. *В исследовании принимали участие 75 больных COVID-19, которые в зависимости от объёма поражения лёгочной паренхимы разделены на две группы в зависимости от объёма поражения паренхимы лёгких. Контрольная группа – практически здоровые люди (n = 24). У всех лиц исследованы количество тромбоцитов в крови и агрегация тромбоцитов, индуцированная аденозиндифосфатом (АДФ), коллагеном и ристомицином; методом полимеразной цепной реакции определяли полиморфизмы rs6065 в гене GP1BA, rs1126643 в гене ITGA2, rs5918 в гене ITGB3.Анализ полученных данных проводили с помощью пакета прикладных программ IBM SPSS Statistics v. 23 (IBM Corp., США).*

Результаты и обсуждение. *У больных с COVID-19-ассоциированным поражением лёгких среднего и тяжёлого течения ускоряется агрегация тромбоцитов, индуцированная АДФ, коллагеном, ристомицином; при тяжёлом течении снижается количество тромбоцитов. Не изменяется частота встречаемости вариантов полиморфизма rs6065, повышается частота встречаемости генотипа T/C полиморфизма rs5918; при средней тяжести повышается частота встречаемости генотипов C/T и T/T полиморфизма rs1126643; при тяжёлом поражении лёгких повышается частота встречаемости мутантного генотипа C/C полиморфизма rs5918. При поражении лёгких средней степени тяжести наличие мутантного варианта T/T полиморфизма rs1126643 ускоряет коллаген-индуцированную агрегацию тромбоцитов; при тяжёлой степени тяжести наличие мутантного C/C и гетерозиготного C/T вариантов полиморфизма rs5918 ускоряет АДФ-индуцированную агрегацию тромбоцитов. Не выявлено влияния полиморфизма rs6065 на агрегацию тромбоцитов. Полученные данные указывают на возможную роль генетической предрасположенности в активации агрегации тромбоцитов у больных с COVID-19-ассоциированным поражением лёгких.*

Ключевые слова: *COVID-19, тромбоциты, агрегация, полиморфизм, GP1BA, ITGA2, ITGB3*

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INTRODUCTION

Changes in hemostasis leading to thrombotic complications are common in hospitalized patients with COVID-19 (coronavirus disease 2019). Given that platelets are key participants and regulators of thrombosis and inflammation, they are an important source of mediators in the pathogenesis of COVID-19 [1, 2]. Patients with COVID-19-associated lung damage have an increased risk of thrombotic complications and mortality, also due to hyperreactivity of platelets [3–5]. Platelets are involved in the pathogenesis of COVID-19 in different ways. SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2) infects bone marrow megakaryocytes; the presence of virions in peripheral blood platelets has been shown to directly increase their aggregation capacity [6]. It has been established that markers of platelet activity (platelet size and maturity) are significantly associated with both disease severity and mortality, even taking into account the presence of comorbidities, medications, and other laboratory parameters, including biomarkers of inflammation and thrombosis (e.g., D-dimer). Platelets in patients with COVID-19 were studied and showed activation of metabolic processes, including oxidative phosphorylation and glycolysis, which increases their aggregation [7]. Multiple platelet activation pathways initiate and maintain thrombus formation. Platelets extracted from patients with COVID-19 show a greater degree of aggregation with various agonists (adenosine triphosphate, adrenaline, collagen, and ristomycin), which may be due to genetic factors [8–10]. A limited number of studies have examined the impact of individual prothrombotic risk factors, both genetic and acquired, on the severity of COVID-19.

Genetic factors include the 4G/5G polymorphism of the plasminogen activator inhibitor-I (PAI-I) gene: it was found to enhance thrombosis-mediated osteonecrosis after COVID-19 infection. A strong correlation between the presence of the C677T polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene and the severity of COVID-19 course has been described [11]. However, the complexity of the clinical course and major complications in patients with severe COVID-19 suggests that a number of other genetic risk factors may be involved in the pathogenesis of COVID-19. This is the reason for the relevance of studies of protein genes involved in hemostasis and cardiovascular complications in patients with COVID-19. These genes include *GP1BA* (glycoprotein Ib-alpha), *ITGB3* (integrin beta 3), and *ITGA2* (integrin alpha 2), which mediate the triggering of various platelet activation mechanisms. The connection of the named genes with increased platelet activation by aggregation inducers in COVID-19 patients has not been systematically studied. The high rate of thrombotic complications in severe forms of COVID-19-associated lung damage may be related to platelet hyperreactivity in conditions of genetic predisposition.

THE AIM OF THE STUDY

To analyze platelet aggregation and polymorphisms in genes providing its realization in patients with COVID-19-associated lung damage, depending on the severity of clinical course.

MATERIALS AND METHODS

The study involved 75 patients with COVID-19 (44 women and 31 men) aged 44 to 75 years, hospitalized in Chelyabinsk Regional Clinical Hospital No. 3, who did not take drugs affecting platelet function before hospitalization and were not related to each other. The control group consisted of 24 clinically healthy volunteers (Group 1), comparable in gender and age to COVID-19 patients and unrelated to each other. Depending on the volume of lung damage, patients with COVID-19 were divided into groups: Group 2 with up to 50 % damage – medium severity (n = 48); Group 3 with more than 50 % damage – severe severity (n = 27), – in accordance with the guidelines of the Ministry of Health of Russia "Prevention, diagnosis and treatment of new coronavirus infection (COVID-19)" [12]. The inclusion criteria for Groups 2 and 3 were the presence of COVID-19 confirmed by detection of SARS-CoV-2 RNA virus on the mucous membranes of the pharynx and nasal cavity using polymerase chain reaction (RealBest SARS-CoV-2 RNA; Vector-Best, Russia). Exclusion criteria were presence of previously detected oncological diseases, chronic diseases of cardiovascular, respiratory, nervous systems and gastrointestinal tract organs; extremely severe course of combined pathology requiring hospitalization of the patient in the intensive care unit; presence of arterial hypertension (stage 2 and higher); body mass index over 30 kg/m^2; anemia. All patients signed an informed consent. The study was approved by the Ethical Committee of the South Ural State Medical University (Protocol No. 4 dated May 25, 2021). In all patients, multispiral computed tomography of the chest (SOMATOM Definition AS 64; Siemens, Germany) revealed bilateral lung damage corresponding to pathognomonic changes in COVID-19: the ground-glass opacity and consolidation type combined with reticular changes.

Blood sampling was performed on day 1 of the patient's admission to the hospital. Standard thromboprophylaxis with unfractionated heparin started after blood sampling for the study. In addition to anticoagulant therapy, patients received standard antiviral therapy and glucocorticosteroids, antibacterial therapy, according to the temporary guidelines of the Ministry of Health of Russia "Prevention, diagnosis and treatment of new coronavirus infection (COVID-19)", revision 15 dated February 22, 2022.

The number of platelets in the blood was counted using Fonio's method. Platelet aggregation was evaluated using a laser platelet aggregation analyzer "ALAT-2" (BIOLA, Russia). Adenosine diphosphate (ADP;

2.5 mmol/ml), collagen (3.3 μg/ml), and ristomycin (7.5 mg/ml) (Technologia Standard, Russia) were used as inducers. The number of aggregates (units) of average size per minute (units/minute) was counted. The average radius of platelets before the onset of aggregation was taken as the unit radius.

Genetic studies were performed using real-time polymerase chain reaction (PCR) (Roche LightCycler 96; Roche Molecular Systems, USA); buccal epithelial scrape was used as the material. We used "SNP-express-cardiogenetics" reagent kits for detection of polymorphisms in genes (Lytech Research and Production Company, Russia). The following polymorphisms were determined: rs6065 (Thr145Met) in the *GP1BA* gene, rs1126643 (Phe253Phe) in the *ITGA2* gene and rs5918 (Leu33Pro) in the *ITGB3* gene. Results were presented as homozygous wild-type variant: C/C for rs6065 and rs1126643 polymorphisms, T/T for rs5918 polymorphism; heterozygous variant: C/T for rs6065 and rs1126643, T/C for rs5918; homozygous mutant variant: T/T for rs6065 and rs1126643, C/C for rs5918.

Statistical processing was performed using IBM SPSS Statistics v. 23 (IBM Corp., USA). Sample characteristics are presented in *Me* (Q_{25}–Q_{75}) format, where *Me* is the median; Q_{25} and Q_{75} are the values of the lower and upper quartiles, respectively. Statistical hypothesis testing in groups was performed using nonparametric Mann – Whitney criteria. Fisher's exact test was used to compare proportions (percentages). Bonferroni correction was introduced in case of multiple comparisons. Differences were considered statistically significant at the level of $p \leq 0.05$. Allele frequencies were estimated using the gene count method, and the χ^2 criterion was used to detect deviations from Hardy – Weinberg equilibrium.

RESULTS

The content of platelets in the blood of patients with moderate lung damage did not change statistically significantly during the study. Group 2 showed statistically significant acceleration of ADP-, collagen-, and ristomycin-induced platelet aggregation by 9 % ($p = 0.038$), 23 % ($p = 0.027$), and 8 % ($p = 0.042$) at the median, respectively, compared to the control group. The number of platelets in blood in patients with severe lung tissue damage was statistically significantly lower by 37 % ($p = 0.002$) at the median compared to the control group. Group 3 showed statistically significant acceleration of ADP-, collagen- and ristomycin-induced platelet aggregation by 21 % ($p = 0.024$), 38 % ($p = 0.003$) and 16 % ($p = 0.019$) respectively compared to the control group. Compared to the group of patients with COVID-19-associated moderate lung damage, the number of platelets in the blood was 30 % lower ($p = 0.007$); ADP-, collagen-, and ristomycin-induced platelet aggregation was accelerated by 16 % ($p = 0.011$), 24 % ($p = 0.004$), and 10 % ($p = 0.009$) at the median, respectively (Fig. 1).

The genotype frequency distribution of *ITGA2*, *GP1BA* and *ITGB3* genes conformed to the expected Hardy – Weinberg equilibrium in both the control group ($p = 0.51$, $p = 0.95$ and $p = 0.81$, respectively) and in the group of patients with COVID-19-associated moderate lung damage ($p = 0.50$, $p = 0.87$ and $p = 0.82$, respectively). The combination of two mutations in one individual was detected in 5 (21 %) patients from the control group and 13 (27 %) patients from Group 2. The combination of three mutations was detected in 1 (4.8 %) patient from the control group and in 4 (8.3 %) patients from Group 2. In the group of patients with COVID-19-associated moderate lung damage the C allele of the rs5918 polymorphism in the *ITGB3* gene is statistically significantly more frequent compared to the control group, and, accordingly, the T allele was less frequent ($p = 0.009$). A lower frequency ($p = 0.012$) of the T/T variant of the rs5918 polymorphic locus of the *ITGB3* gene (64.5 % of observations) and a higher frequency ($p = 0.007$) of the T/C variant (29.2 % of observations) were determined in Group 2 compared to the control group. The frequency of the C/C variant did not change statistically significantly. No statistically significant differences with Group 1 were found in Group 2, when analyzing the frequency of alleles and genotypes of the rs6065 polymorphic locus in the *GP1BA* gene, including the C/C mutant variant. When the frequency of the rs1126643 polymorphism in the *ITGA2* gene was analyzed, it was found that the C allele was less frequent ($p = 0.022$), and the T allele was more frequent ($p = 0.014$) than the corresponding alleles in the control group. The C/C variant of this polymorphism was found in 39.6 % of observations, statistically significantly less frequent ($p = 0.002$) than in the control group. C/T and T/T variants were found in 43.8 and 16.6 % of observations, respectively, which was more frequent ($p = 0.031$ and $p = 0.042$, respectively) than in the control group (Table 1).

The genotype frequency distribution of the *ITGA2*, *GP1BA*, and *ITGB3* genes in Group 3 was consistent with the expected Hardy – Weinberg equilibrium ($p = 0.47$, $p = 0.82$, and $p = 0.71$, respectively). The combination of two mutations was found in 6 (22.2 %) patients, three mutations – in 3 (11 %) people. When analyzing the rs5918 polymorphism of the *ITGB3* gene, the C allele was statistically significantly more frequent and, accordingly, the T allele was less frequent both in comparison with the control and in comparison with Group 2 (Table 1). The frequency of T/T variant of the rs5918 polymorphic locus of the *ITGB3* gene in Group 3 was 51.9 %, which was statistically significantly lower than in Groups 1 and 2 ($p = 0.030$ and $p = 0.038$, respectively), while T/C variant was detected in 29.6 % of observations, which was statistically significantly higher than in the control group, but did not differ from the values of Group 2. The frequency of the C/C mutant variant in this group was 18.5 %, which was statistically significantly higher ($p = 0.043$) compared to the control group and Group 2. When analyzing the frequency distribution of alleles and genotypes of the rs6065 polymorphic locus in the *GP1BA* gene in Group 3, no statistically significant differences with the control group

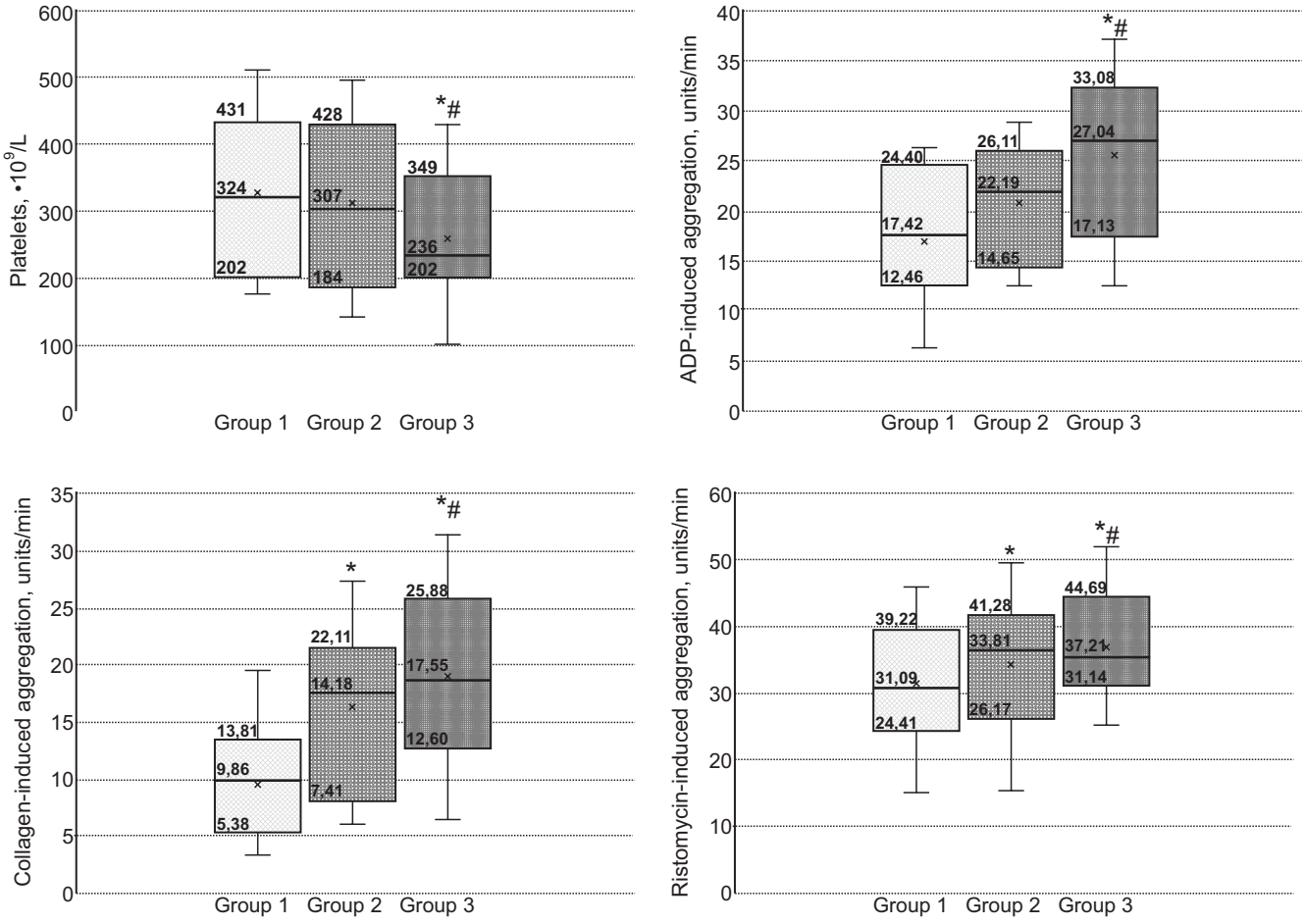

FIG. 1.

*Platelet blood count and their aggregation rate in COVID-19-associated lung damage depending on the severity of the disease. ——— – median; ▭ – 25th–75th percentiles; × – arithmetic mean; * – statistically significant ($p < 0.05$) differences with Group 1; # – statistically significant ($p < 0.05$) differences with Group 2*

and with Group 2 were found. When analyzing the frequency of the rs1126643 polymorphism in the *ITGA2* gene, no differences with the control group were found, but it was found that the C allele was more frequent, and the T allele was less frequent than the corresponding alleles in Group 2. The C/C variant of the rs1126643 polymorphism was found in 59.3 % of cases, which did not differ from the control group but was statistically significantly more frequent than in Group 2 ($p = 0.024$). The C/T variant was found in 29.6 % of observations, which was less frequent ($p = 0.033$) than the same variant in Group 2 and not statistically significantly different from the control group. The T/T variant was found in 11.1 % of cases, which was not statistically significant when compared with Groups 1 and 2.

During the analysis of platelet aggregation in Group 2 depending on the rs1126643 polymorphism of the *ITGA2* gene, it was noted that the T/T mutant variant accelerated collagen-induced platelet aggregation by 31 % ($p = 0.011$) and 23 % ($p = 0.019$) compared to the C/C and C/T variants, respectively. Statistical analysis revealed no differences in induced platelet aggregation in this group depending on polymorphisms of *ITGB3* and *ITGA2* genes (Table 2).

As a result of platelet aggregation analysis in Group 3 in the presence of the rs5918 polymorphism of the *ITGB3* gene, it was noticed that a statistically significant acceleration of ADP-induced platelet aggregation by 13 % ($p = 0.022$) was found in the T/C variant compared to the T/T variant. In the C/C variant, ADP-induced platelet aggregation was accelerated by 18 % ($p = 0.017$) compared to the T/T variant and did not change compared to the T/C variant. Statistical analysis revealed no statistically significant connections of other studied genotypes of the *ITGB3* and *ITGA2* genes with platelet aggregation in Group 3. The study showed no effect of the rs6065 polymorphism of the *GP1BA* gene on platelet aggregation ability.

DISCUSSION

Decrease in the number of platelets in patients with COVID-19-associated lung damage is present in 58–

TABLE 1

FREQUENCY OF OCCURRENCE OF GENOTYPES IN THE ANALYSIS OF *ITGB3*, *GP1B1*, AND *ITGA2* GENE POLYMORPHISMS IN COVID-19-ASSOCIATED LUNG DAMAGE, Me (Q25; Q75)

Polymorphisms	Genotypes/alleles		Genotype frequency		
			Group 1 (n = 24)	Group 2 (n = 48)	Group 3 (n = 27)
rs5918 in the *ITGB3* gene	genotypes	*0 (T/T)*	19 (79.0 %)	**31 (64.5 %)***	**14 (51.9 %)*#**
		I (T/C)	4 (16.7 %)	**14 (29.2 %)***	**8 (29.6 %)**
		II (C/C)	1 (4.3 %)	3 (6.3 %)	**5 (18.5 %)*;#**
	alleles	*T*	87.3 %	**79.1 %***	**66.7 %*;#**
		C	12.7 %	**20.9 %***	**33.3 %*;#**
rs6065 in the *GP1BA* gene	genotypes	*0 (C/C)*	20 (83.4 %)	38 (79.1 %)	22 (81.4 %)
		I (C/T)	4 (16.6 %)	9 (18.8 %)	5 (18.6 %)
		II (T/T)	0	1 (2.1 %)	0
	alleles	*C*	91.7 %	88.5 %	90.7 %
		T	8.3 %	11.5 %	9.3 %
rs1126643 in the *ITGA2* gene	genotypes	*0 (C/C)*	16 (66.7 %)	**19 (39.6 %)***	**16 (59.3 %)#**
		I (C/T)	6 (25.0 %)	**21 (43.8 %)***	**8 (29.6 %)#**
		II (T/T)	2 (8.3 %)	**8 (16.6 %)***	3 (11.1 %)
	alleles	*C*	79.2 %	**61.5 %***	**74.1 %#**
		T	20.8 %	**38.5 %***	**25.9 %#**

Note. * – statistically significant ($p < 0.05$) differences with Group 1; # – statistically significant ($p < 0.05$) differences with Group 2; 0 – homozygous wild-type variant; I – heterozygous variant; II – homozygous mutant variant..

TABLE 2

THE RATE OF PLATELET AGGREGATION IN COVID-19-ASSOCIATED LUNG DAMAGE DEPENDING ON POLYMORPHISMS OF *ITGB3*, *GP1B1* AND *ITGA2* GENES, Me (Q25; Q75)

Polymorphisms	Genotypes	Group 2 (n = 48)	Group 3 (n = 27)
	ADP-induced aggregation (units/min)		
rs5918 in the *ITGB3* gene	*0 (T/T)*	19.84 (14.45; 23.32)	20.19 (17.11; 23.17)
	I (T/C)	20.71 (15.81; 25.02)	**23.19 (17.31; 25.72)***
	II (C/C)	21.22 (18.35; 24.11)	**24.74(16.91; 28.12)***
	Ristomycin-induced aggregation (units/min)		
rs6065 in the *GP1BA* gene	*0 (C/C)*	33.72 (26.17; 39.03)	38.58 (31.14; 44.69)
	I (C/T)	34.06 (28.44; 40.28)	36.41 (32.78; 39.90)
	II (T/T)	27.18	–
	Collagen-induced aggregation (units/min)		
rs1126643 in the *ITGA2* gene	*0 (C/C)*	13.17 (7.31; 19.63)	17.17 (12.60; 24.11)
	I (C/T)	14.02 (8.67; 24.15)	18.24 (13.41; 22.58)
	II (T/T)	**17.25 (9.42; 22.14)*;#**	16.22 (13.01; 19.28)

Note. * – statistically significant ($p < 0.05$) differences with homozygous wild-type variant within a group; # – statistically significant ($p < 0.05$) differences with heterozygous variant within a group; 0 – homozygous wild-type variant; I – heterozygous variant; II – homozygous mutant variant.

95 % of cases and can be associated with multiple factors. Thrombocytopenia (low platelet count) in the early stages of COVID-19 is usually due to platelet destruction and increased platelet consumption, but decreased platelet production may be evident in the later stages of the disease [9]. Previously, an association between blood platelet count, severity of disease course, and increased risk of mortality in hospitalized patients with COVID-19 has been demonstrated [13]. In COVID-19, platelet activation is carried out by inflammatory mediators, antigen-antibody complexes, and damaged endothelium. The resulting activated platelets are removed from the bloodstream by mononuclear phagocytes. SARS-CoV-2 is able to inhibit thrombopoiesis through direct interaction with megakaryocytes and also has a direct effect on platelet through binding to ACE2 receptors, which causes escalation of oxidative stress in platelets, their increased consumption, impaired thrombopoiesis in bone marrow, triggering and autoimmune reactions [14]. The products of cells (primarily endotheliocytes) destroyed by SARS-CoV-2 may be another cause of accelerated platelet aggregation, which leads to thrombosis and thrombocytopenia. Individual genotype characteristics, including polymorphisms of the *GP1BA*, *ITGA2*, and *ITGB3* genes, are other factors that have a prominent effect on platelet aggregation activity.

The *GP1BA* gene encodes the α-subunit of glycoprotein Ib involved in the formation of the platelet receptor GpIb/IX/V. The main ligand of the receptor is von Willebrand factor (vWF), which binds platelets to the site of vascular damage [15]. The strength of the binding formed between them depends largely on the configuration of the receptor, vWF structure and blood flow velocity. The rs6065 polymorphism of the *GP1BA* gene is connected with the cytosine(C)-to-thymidine(T) substitution near the initiation of genetic transcription, which results in threonine-to-methionine substitution in the receptor site responsible for binding to vWF. As a result, carriers of the C/C genotype have a higher concentration of platelet membrane glycoprotein Ib than those with other genotype variants. The mutant homozygous T/T form (frequency in the population is about 1.5 %) sharply increases the risks of thrombosis. In case of heterozygous C/T variant, the expression of GpIb/IX/V receptors on platelets is not so strongly expressed, but some studies have revealed an increased risk of thrombosis in carriers of this gene variant [16]. Several studies show that carriers of the C allele have an increased risk of coronary thrombosis, ischemic stroke, and a decreased age of its onset [17].

In the presented study, there are no data indicating an effect of the rs6065 polymorphism of the *GP1BA* gene on the acceleration of ristomycin-induced platelet aggregation, reflecting the interaction of vWF with the GpIb receptor. A possible explanation is the absence of a significant number of carriers of the homozygous mutant variant of the T/T gene in the study population and small sample size. In addition, there is a cooperativity effect in the binding of vWF to platelet GpIb: glycoproteins IX and V, whose structure and function may remain intact, participate in the formation of collagen complex. In addition, the GpIb/IX/V receptor serves more for platelet adhesion. Its role in aggregation is less prominent [18]. Thus, the acceleration of ristomycin-induced platelet aggregation in patients with COVID-19-associated lung damage cannot be explained by the *GP1BA* gene rs6065 polymorphism. It is probably influenced by other genetic and non-genetic factors.

The *ITGB3* gene regulates the synthesis of the membrane protein integrin β-3, which is involved in interplatelet interactions. Integrin β-3 is a component of glycoprotein IIb/IIIa and recognizes the specific amino acid sequence – glycine-proline-arginine – in a wide range of ligands including prothrombin, fibrinogen, plasminogen, and vWF. Integrin β-3 is a heterodimer composed of noncovalently associated α- and β-subunits. These subunits have a large extracellular part, a transmembrane part and a short cytoplasmic part [19]. The rs5918 polymorphism of the *ITGB3* gene is caused by the thymine (T) to cytosine (C) nucleotide substitution in a certain DNA region, which results in the leucine amino acid-to-proline substitution in position 33 of the protein chain and disruption of the three-dimensional structure of the receptor. Disruption of the receptor structure leads to increased platelet reactivity and contributes to their thrombogenicity [20, 21]. Individuals with the C/C variant of this polymorphism have an increased propensity for platelet aggregation and, consequently, the risk of thrombosis [22]. It is assumed that the effect of the rs5918 polymorphism on platelet characteristics is found only for homozygotes for the C allele [23].

The association of the rs5918 polymorphism of the *ITGB3* gene with the occurrence of thrombotic events has been previously recognized [22–24]. Based on the presence of these adverse events in patients with COVID-19 and the frequency of T/C and C/C variants of the rs5918 polymorphism in these patients, it can be assumed that this genetic defect is of significance in the pathogenesis of COVID-19, especially in disorders of the hemostasis system. We found that the frequency of heterozygotes and mutant homozygotes of the *ITGB3* gene was higher in patients with accelerated ADP-induced aggregation as well as severe COVID-19. Increased mean platelet volume and increased concentration of glycoprotein IIb/IIIa on the platelet membrane are characteristic of mutant homozygote carriers and, to a lesser extent, heterozygotes of the rs5918 polymorphism. As a consequence of such changes, platelet reactivity is increased [25]. However, the exact mechanisms that cause platelet aggregation in the presence of the C/C variant of the rs5918 polymorphism are not thoroughly understood and require further study.

The *ITGA2* gene encodes the protein integrin alpha-2, a membrane glycoprotein GPIa found on the membranes of various cells, including platelets. On the platelet membrane, GPIa forms a complex with GPIIa, which is one of the collagen receptors. The rs1126643 polymorphism of the *ITGA2* gene is caused by the cytosine(C)-to-thymidine(T) substitution. This mutation alters the amino acid sequence, which leads to a correlation between this polymorphism and the level of GPIa expression

on the platelet membrane [26]. In the case of the T/T variant of the rs1126643 polymorphism, platelets bind to collagen more rapidly. Heterozygous individuals with the C/T variant show an intermediate level of receptor expression [27]. The data obtained in this study (acceleration of collagen-induced platelet aggregation) are consistent with the existing data on the function of integrin alpha-2 and may suggest a role for the rs1126643 polymorphism in the pathogenesis of COVID-19, although its mechanism remains unclear.

The obtained data suggest a possible role of genetic predisposition, in particular polymorphisms (rs6065 of *GP1BA* gene, rs1126643 of *ITGA2* gene and rs5918 of *ITGB3* gene), in platelet hyperactivation in patients with COVID-19-associated lung damage. These findings can be used to plan larger studies in order to assess the potential risk of thrombotic complications, to determine further treatment tactics and the choice of anticoagulant and disaggregant therapy. Hemostasis links are controlled by different genes, and the influence of their polymorphisms on the pathogenesis of COVID-19 has not been sufficiently investigated. The association of genetic factors with possible complications and severity of the disease course requires further research.

CONCLUSIONS

Induced platelet aggregation is accelerated in patients with COVID-19-associated lung damage of moderate and severe course. In severe course, the number of platelets in the blood decreases. The frequency of heterozygous C/T and homozygous mutant T/T genotypes of the rs1126643 polymorphism of the *ITGA2* gene is increased in moderate severity of lung damage. The presence of T/T genotype is associated with collagen-induced platelet aggregation. In severe lung damage the frequency of homozygous mutant C/C genotype of the rs5918 polymorphism of the *ITGB3* gene is increased. The presence of C/C and C/T genotypes of the rs5918 polymorphism is associated with ADP-induced platelet aggregation.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Mikhail V. Osikov – Dr. Sc. (Med.), Professor, Head of the Department of Pathophysiology, South Ural State Medical University; Head of the Research Department, Chelyabinsk Regional Clinical Hospital, e-mail: prof.osikov@yandex.ru, https://orcid.org/0000-0001-6487-9083

Vladimir N. Antonov – Dr. Sc. (Med.), Professor at the Department of Therapy, Institute of Continuing Professional Education, South Ural State Medical University; Head of the Regional Pulmonology Center, Chelyabinsk Regional Clinical Hospital, e-mail: ant-vn@yandex.ru, https://orcid.org/0000-0002-3531-3491

Semen O. Zotov – Teaching Assistant at the Department of Pathophysiology, South Ural State Medical University; Head of the Infectious Diseases Department No. 5, Regional Center for Infectious Diseases, Regional Clinical Hospital No. 3, e-mail: semenz2007@yandex.ru, https://orcid.org/0000-0001-7469-2386

INFECTIOUS DISEASES

THE rs11385942 AND rs657152 VARIANTS ARE NOT ASSOCIATED WITH COVID-19 SEVERITY AND OUTCOMES IN PATIENTS TREATED WITH FAVIPIRAVIR AND REMDESIVIR

Abdullaev Sh.P. [1],
Denisenko N.P. [1],
Temirbulatov I.I. [1],
Kachanova A.A. [1],
Tuchkova S.N. [1],
Mikhaylenko E.V. [2],
Kryukov A.V. [1],
Valiev T.T. [1],
Mirzaev K.B. [1],
Sychev D.A. [1]

[1] Russian Medical Academy of Continuous Professional Education (Barrikadnaya str. 2/1, building 1, Moscow 125993, Russian Federation)
[2] I.M. Sechenov First Moscow State Medical University (Sechenov University) (Trubetskaya str. 8, building 2, Moscow 119991, Russian Federation)

Corresponding author:
Sherzod P. Abdullaev,
e-mail: abdullaevsp@gmail.com

ABSTRACT

Background. *There is a mounting evidence in the scientific literature that susceptibility to SARS-CoV-2 infection could vary. The severity of COVID-19 symptoms can range from asymptomatic to severe respiratory failure, requiring prolonged artificial ventilation. The underlying causes of this range of clinical manifestations remain unclear. Identification of the risk factors that may cause this variation in clinical symptoms is important for identifying the most susceptible populations at highest risk. This should help improve prevention measures, reduce hospitalizations, and decrease the mortality rate of the disease. Previously, an association has been found between the severity of COVID-19 and the genetic markers rs11385942 G>GA and rs657152 A>C.*

The aim. *To assess the impact of carrying polymorphic markers rs11385942 G>GA and rs657152 A>C on the severity of COVID-19 in patients undergoing specific therapy.*

Materials and methods. *A total of 240 patients hospitalized with a coronavirus infection were included in the study. All patients received therapy with favipiravir or remdesivir. The presence of the rs11385942 G>GA and rs657152 A>C variants was determined in all patients. The study compared the length of hospital stays, frequency of patient transfers to the intensive care unit (ICU), and frequency of clinical outcomes (recovery or death) among carriers of allelic variants of the markers under investigation.*

Results. *There were no significant associations between the carriage of variants rs11385942 G>GA and rs657152 A>C and the duration of patients' hospitalization, frequency of patient transfers to the ICU, and patient outcomes.*

Conclusion. *The carriage of rs11385942 G>GA and rs657152 A>C variants did not affect the severity or type of clinical outcomes in patients with COVID-19.*

Key words: *susceptibility to COVID-19, COVID-19 severity, etiotropic therapy of COVID-19, favipiravir, remdesivir, rs11385942, rs657152, polymorphisms*

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ВАРИАНТЫ rs11385942 И rs657152 НЕ АССОЦИИРУЮТСЯ С ТЯЖЕСТЬЮ ТЕЧЕНИЯ И ИСХОДАМИ COVID-19 У ПАЦИЕНТОВ, ПОЛУЧАВШИХ ТЕРАПИЮ ФАВИПИРАВИРОМ И РЕМДЕСИВИРОМ

Абдуллаев Ш.П. [1],
Денисенко Н.П. [1],
Темирбулатов И.И. [1],
Качанова А.А. [1],
Тучкова С.Н. [1],
Михайленко Е.В. [2],
Крюков А.В. [1],
Валиев Т.Т. [1],
Мирзаев К.Б. [1],
Сычев Д.А. [1]

[1] ФГБОУ ДПО «Российская медицинская академия непрерывного профессионального образования» Минздрава России (125993, г. Москва, ул. Баррикадная, 2/1, стр. 1, Россия)
[2] ФГАОУ ВО «Первый Московский государственный медицинский университет им. И.М. Сеченова» Минздрава России (Сеченовский университет) (119991, г. Москва, ул. Трубецкая, 8, стр. 2, Россия)

Автор, ответственный за переписку:
Абдуллаев Шерзод Пардабоевич,
e-mail: abdullaevsp@gmail.com

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РЕЗЮМЕ

Обоснование. *В научной литературе появляется всё больше данных о различиях в чувствительности и восприимчивости к инфекции SARS-CoV-2, которые проявляются у пациентов в диапазоне от бессимптомного течения заболевания до тяжёлой дыхательной недостаточности и необходимости длительной искусственной вентиляции лёгких. Основные причины этого спектра клинических проявлений остаются неясными. Определение факторов риска, способных вызвать такую вариацию клинических симптомов, важно для выявления наиболее восприимчивых групп населения с наибольшим риском. Это должно помочь улучшить меры профилактики, сократить количество госпитализаций и снизить смертность от заболевания. Ранее для генетических маркеров rs11385942 G>GA и rs657152 A>C была показана связь с тяжестью течения COVID-19.*

Цель работы. *Оценить вклад носительства полиморфных маркеров rs11385942 G>GA и rs657152 A>C на показатели тяжести течения COVID-19 у пациентов, получавших этиотропную терапию.*

Материалы и методы. *В исследование было включено 240 пациентов, госпитализированных в ГБУЗ г. Москвы «Городская клиническая больница № 15 им. О.М. Филатова ДЗМ» с диагнозом COVID-19, получавших этиотропную терапию фавипиравиром или ремдесивиром. У всех пациентов определялось носительство вариантов rs11385942 G>GA и rs657152 A>C. Сравнивались длительность стационарного лечения, частота перевода пациентов в отделение реанимации и интенсивной терапии (ОРИТ), частота наступления клинических исходов (выписан или смерть) между носителями аллельных вариантов изучаемых генетических маркеров.*

Результаты. *Не было выявлено статистически значимых ассоциаций носительства различных вариантов rs11385942 G>GA и rs657152 A>C с длительностью госпитализации пациентов, частотой перевода пациентов в ОРИТ и наступлением того или иного исхода.*

Заключение. *Носительство вариантов rs11385942 G>GA и rs657152 A>C не определяло показатели тяжести течения и вид клинических исходов у пациентов с COVID-19.*

Ключевые слова: *восприимчивость к COVID-19, тяжесть течения COVID-19, этиотропная терапия COVID-19, фавипиравир, ремдесивир, rs11385942, rs657152, полиморфизмы*

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INTRODUCTION

The COVID-19 pandemic has had a significant impact on health systems in nearly every country in the world. The experience of coping with the COVID-19 crisis revealed many shortcomings in the organization of medical service and public drug supply. In the initial phase of the fight against the disease, the scientific community studied the possibilities of using already known drugs. Thus, in spring 2020, favipiravir and remdesivir were already proposed as treatment options for COVID-19 [1, 2]. According to the latest version of the Provisional Guidelines for the Prevention, Diagnosis and Treatment of COVID-19, both drugs are categorized as drugs for etiotropic therapy of COVID-19 and remain relevant [3].

There is increasing evidence in scientific literature of differences in sensitivity and susceptibility to SARS-CoV-2 infection, manifesting in a range from asymptomatic disease course to severe respiratory failure and the need for prolonged artificial lung ventilation (ALV) [4]. The underlying causes of this spectrum of clinical outcomes remain unclear. Identification of risk factors, such as genetic, clinical-demographic, environmental, and possible other factors that may cause this variation in clinical symptoms is important to identify the most susceptible populations at highest risk. This should protect them from infection, reduce hospitalizations and reduce mortality.

Previously, a group of scientists suggested that the severity of the course of COVID-19 may, among other things, be determined by the genetic profile of patients. D. Ellinghaus et al. (2020) in their work identified associations of carrying allelic variants of markers *rs11385942 G>GA* at locus 3p21.31 and *rs657152 C>A* at locus 9q34.2 with severe forms of respiratory failure in COVID-19 patients [5]: GWAS study showed that the signal at locus 3p21.31 covered the *SLC6A20*, *LZTFL1*, *CCR9*, *FYCO1*, *CXCR6* and *XCR* genes, while the association signal at locus 9q34.2 coincided with the ABO blood group locus. Based on these results, the authors concluded the possible role of the 3p21.31 gene clusters and ABO blood group as a predictor of COVID-19 susceptibility in patients with respiratory failure [5]. The impact of carrying these markers on the effectiveness of therapy in COVID-19 patients has not been previously studied.

Taking into account the abovementioned, the aim of the presented study was a comparative assessment of the distribution of carriage of allelic variants *rs11385942 G>GA* and *rs657152* in groups of patients with COVID-19, differing in the duration of hospitalization, frequency of transfer to the intensive care unit (ICU) and clinical outcomes of the disease treatment.

MATERIALS AND METHODS

The study was conducted at the Moscow City Clinical Hospital No. 15 named after O.M. Filatov Moscow Health Department and was approved by the local ethical committee of the Federal State Budgetary Educational Institution of Professional Education "Russian Medical Academy of Continuing Professional Education" of the Ministry of Health of Russia (Minutes No. 15 dated October 16, 2021). Voluntary informed consent was obtained from all patients for participation in this study.

Study sample characteristics

The authors performed a prospective observational unmasked study. The study was conducted from November 2021 through February 2022. The study included 240 patients, male and female, aged 18 years and older, hospitalised with confirmed new coronavirus infection (COVID-19) (U07.1, U07.2 according to ICD), complying with inclusion criteria and failing non-inclusion criteria. The age of all patients ranged from 44 to 96 years (mean age – 73.0 ± 12.5 years). Of these, 74 (31 %) were men (mean age – 72.91 ± 12.62 years) and 166 (69 %) were women (mean age – 73.0 ± 12.5 years).

Inclusion criteria were confirmed diagnosis of new COVID-19 coronavirus infection (U07.1, U07.2 according to ICD); duration of hospitalisation > 48 h; use of favipiravir and remdesivir as etiotropic therapy; and signed voluntary informed consent.

Non-inclusion criteria were contraindications to aetiotropic therapy: severe hepatic insufficiency (Child – Pugh class C); glomerular filtration rate (GFR) < 30 ml/min/1.73 m^2; pregnancy and breastfeeding.

Patients received favipiravir and remdesivir as etiotropic therapy. Remdesivir was used in the standard dosage – 200 mg intravenously on the first day, then 100 mg once daily for 5–10 days. The dosing regimen of favipiravir was selected cause-specific to the patient's weight according to the instructions for medical use: for patients with body weight less than 75 kg – 1600 mg 2 times a day on the first day, further – 600 mg 2 times a day; for patients with body weight more than 75 kg – 1800 mg 2 times a day on the first day, further – 800 mg 2 times a day. The surveyor could not influence the choice of antiviral drug and the duration of therapy, which were determined by the treating physician.

Genotyping

After obtaining written informed consent and inclusion in the study, 10 ml of venous blood was sampled from each patient for subsequent genetic testing. Genomic DNA was isolated from whole blood using S-Sorb reagent kits (OOO "Syntol", Russia). The concentration of extracted DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The carriage of allelic variants *rs11385942 G>GA* and *rs657152 A>C* was determined by the allele-specific polymerase chain reaction (PCR) in real time on the CFX96 Touch Real Time System (BioRad, USA) using commercial kits TaqMan®SNP Genotyping Assays and TaqMan Universal MasterMix II, no UNG (Applied Biosystems, USA) according to the manufacturer's instructions (Table 1). The amplification mode was: 95 °C for 5 min; 40 cycles, 95 °C for 10 s, 55 °C for 10 s; 72 °C for 10 s.

Statistical processing

Consistency of genotype frequencies with the Hardy – Weinberg equilibrium was assessed using Pearson's χ^2 (chi-square) criterion (equilibrium is satisfied at $p > 0.05$). Fisher's exact test was used to assess differences in the frequencies of occurrence of different alleles between groups.

The normality of the obtained results distribution was assessed using the Shapiro – Wilk W-test and the Kolmogorov – Smirnov criterion. Categorical variables were compared using Pearson's χ^2 test or Fisher's two-sided exact test (depending on the nature of the distribution of indicators). Multiple samples of continuous data were compared using one- or multivariate analysis of variance ANOVA or Kraskell – Wallis H-test (depending on the nature of data distribution).

IBM SPSS Statistics 22 software package (IBM Corp., USA) was used as a means of statistical processing. For all results, a value of $p < 0.05$ was considered statistically significant.

RESULTS

The severity of the course and outcome of the disease were assessed in groups with respect to the carriage of alleles of the studied polymorphic markers *rs11385942 G>GA* and *rs657152 A>C*. The following indices were used for analysis: 1) duration of COVID-19 patients' hospital stay; 2) frequency of patients' transfer to ICU; 3) outcome of the disease treatment in the form of discharge, transfer of the patient from the infectious diseases department to other departments and patient's death.

Correlations between the frequency of transfer to ICU, frequency of deaths, severity of course, sex and age of patients, level of comorbidity and concomitant therapy in the treatment groups were not analysed, which is a limitation of the conducted analysis.

Among all patients, the genotype distribution of markers *rs11385942 G>GA* and *rs657152 A>C* agreed with the Hardy – Weinberg law ($p > 0.05$) (Table 2).

Analysis of the correlation between the carriage of *rs11385942 G>GA* variants and the number of days the patient spent in hospital (bed-days) revealed no differences ($p = 0.8335$). Similar conclusions can be drawn for *rs657152 A>C*: there were no statistically significant differences between carriers of different genotypes ($p = 0.9693$) (Table 3). Carriage of *rs11385942 G>GA* and *rs657152 A>C* variants did not determine the length of hospitalization of COVID-19 patients.

In 75 patients included in the study, the disease was severe, which required transfer of these patients to ICU. Fisher's test showed that death was statistically significantly more frequent among patients who were admitted to ICU than among patients who were not admitted to ICU ($p < 0.0001$). When comparing the frequency of patients admitted to ICU cause-specific to *rs11385942 G>GA* and *rs657152 A>C* carrier variants, no statistically significant differences were also revealed (Table 4).

TABLE 1
USED PRIMERS

SNP	RefSeq	Primer F 5'-3'	Primer R 5'-3'
LZTFL1 *rs11385942*	NM_020347.4	TGGGGCTAGTGTGTGAGGA	AGCACCACCTTCTCAGAGTT
ABO *rs657152*	NM_020469.3	TCCTACGGGAGGCAGCAGT	AATTTAGGACATGTAAAGTTCA

Note. According to the ThermoFisher manufacturer's instructions (https://www.thermofisher.com/taqman/results?keyword=).

TABLE 2
GENOTYPE AND ALLELE FREQUENCIES OF *rs11385942 G>GA* AND *rs657152 A>C* ALLELIC VARIANTS IN THE GROUP

Markers	N		Genotypes, *n* (%)			Alleles, *n* (%)		Conformity to the Hardy – Weinberg distribution*	
			GG	G/GA	GA	G	GA	χ^2	p
rs11385942 G>GA	240	observed	162	72	6	396 (82.50)	84 (17.50)	0.3643	0.8335
		expected	163.4	69.3	7.4				
		%	67.5	30.0	2.5				
			AA	AC	CC	A	C		
rs657152 A>C	240	observed	49	121	70	219 (45.63)	261 (54.38)	0.0623	0.9693
		expected	50.0	119.1	71.0				
		%	20.4	50.4	29.2				

Note. * – Pearson's χ^2 criterion.

As mentioned above, patient discharge from the hospital, patient transfer to another department of the hospital and recording of patient death in the department were used as parameters to assess patient outcome in the Infectious Diseases Department.

A total of 41 deaths were recorded in the study group, accounting for 17.1 %. 165 (68.7 %) patients were discharged from the hospital, and 34 (14.2 %) patients were transferred to other hospital departments. When a patient was transferred to another hospital department, no further follow-up by the surveyor was conducted; therefore, such patients were excluded from the analysis of treatment outcomes in relation to marker carriage. Comparison of the number of discharged and deceased patients caused-specific to carriage of *rs11385942 G>GA* and *rs657152 A>C* variants revealed no statistically significant association ($p > 0.05$) (Table 5).

In case of transfer to another department, clinical outcome was not recorded in these patients, which is also one of the limitations of the study.

DISCUSSION

COVID-19 is an acute viral disease, causing predominant involvement of the respiratory tract, the course of which can vary from asymptomatic and mild (in most cases) to life-threatening conditions with the development of severe respiratory failure and acute respiratory distress syndrome (up to 5 %) [6, 7]. COVID-19 mortality was relatively higher among patients with severe disease and those treated in the ICU [8, 9]. It appears that the high mortality rate is primarily related to the manifestations of severe respiratory failure requiring transfer of patients to the intensive care unit [10].

TABLE 3

ASSOCIATION BETWEEN THE DURATION OF PATIENTS' PERIOD OF HOSPITALIZATION AND CARRIAGE OF *rs11385942 G>GA* AND *rs657152 A>C* VARIANTS

Markers	*N*	Genotypes	Number of bed days		*p**
			Mean (± SD)	Median bed days [Q1–Q3]	
rs11385942 G>GA	240	GG	11.41 (± 8.79)	9.0 [6.0–14.0]	0.2835
		G|GA	13.08 (± 8.99)	10.0 [7.0–16.0]	
		GA|GA	9.83 (± 5.42)	9.5 [9.0–10.0]	
rs657152 A>C	240	AA	12.06 (± 7.85)	9.0 [7.0–15.0]	0.6831
		AC	11.39 (± 7.73)	9.0 [6.0–13.0]	
		CC	12.59 (± 10.96)	10.0 [7.0–14.0]	

Note. SD – standard deviation; * – Kraskell – Wallis H-test.

TABLE 4

ASSOCIATION BETWEEN THE FREQUENCY OF TRANSFER OF PATIENTS TO INTENSIVE CARE UNIT AND CARRIAGE OF *rs11385942 G>GA* AND *rs657152 A>C* VARIANTS

Markers	*N*	Genotypes	Number of patients outside ICU, *n* (%)	Number of patients transferred to ICU, *n* (%)	χ^2	*p**
rs11385942 G>GA	240	GG	115 (47.9)	47 (19.6)	1.18159	0.553886
		G|GA	46 (19.2)	26 (10.8)		
		GA|GA	4 (1.7)	2 (0.8)		
rs657152 A>C	240	AA	33 (13.8)	16 (6.7)	0.255425	0.880106
		AC	85 (35.4)	36 (15)		
		CC	47 (19.6)	23 (9.6)		

Note. * – Pearson's χ^2 criterion.

TABLE 5

ASSOCIATION BETWEEN THE DEATH RATE AND PATIENT HOSPITAL DISCHARGE AND CARRIAGE OF *rs11385942 G>GA* AND *RS657152 A>C* VARIANTS

Markers	*N*	*n*	Genotypes	Treatment outcomes		χ^2	*p**
				Death, *n* (%)	Discharged, *n* (%)		
rs11385942 G>GA	206	138	GG	28	110	0.041900	0.979268
		63	G|GA	12	51		
		5	GA|GA	1	4		
rs657152 A>C	206	40	AA	7	33	1.21099	0.545803
		110	AC	25	85		
		56	CC	9	47		

Note. * – Pearson's χ^2 test.

Patient genetic characteristics may also contribute to the severity of the COVID-19 course. In the GWAS study by D. Ellinghaus et al. it was revealed that the frequency of carrying the risky GA allele of the *rs11385949 G>GA* marker was higher among patients who developed acute respiratory failure during COVID-19 and were placed on artificial ventilation compared to those who received only oxygen support [5]. The explanation for this association of the *rs11385949 G>GA* variant was that the region near *rs11385942* on chromosome 3p21.31 significantly affects the expression of the *LZTFL1* gene ($p < 0.05$), a regulator of the cilia of the respiratory tract [11]. The second identified risk marker for COVID-19 severity *rs657152 A>C* was more frequent in patients with COVID-19 and development of respiratory failure. This marker matched the locus coding for ABO system blood groups [5].

In the study of O. Balanovsky et al. (2021) the distribution frequencies of *rs11385942 G>GA* and *rs657152 A>C* among populations living in Russia and bordering countries were studied. Analysis of the correlation between the frequency of carriage of these markers and COVID-19 mortality rates revealed a positive association. The correlation was stronger for *rs657152 A>C* ($r = 0.59$; $p = 0.02$). The authors pointed out that such a correlation was revealed only for the Russian sample and was not relevant for data for global populations [12].

In this study, we attempted to assess the putative impact of carriage of COVID-19 severity markers over the length of hospital stay, the frequency of transfer of such patients to the ICU, and the final therapy outcomes. Carriage of the studied markers *rs11385942 G>GA* and *rs657152 A>C* was not associated with prolongation of the patient's length of hospital treatment, had no statistically significant effect on the proportion of patients transferred to ICU, and was not associated with a statistically significant difference in mortality between patients with different genotypes. Our data are consistent with the findings of other studies. For instance, E.A. Orlova et al. analysed the frequency of *rs657152 A>C* in cohorts of 129 COVID-19 patients and 466 healthy individuals and found no statistically significant differences between them. The distribution of *rs657152 A>C* frequencies between patients with high and low viral loads revealed no differences. The authors therefore concluded that carriage of *rs657152 A>C* alone cannot be considered a risk factor for a more severe course of COVID-19 [13]. Similar findings about the lack of association between the carriage of the markers in question and the severity of the disease course were obtained in a case-control study by R. Marçalo et al. When comparing patients with chronic obstructive pulmonary disease (COPD) ($n = 255$) and patients without COPD ($n = 243$) in terms of COVID-19 course severity and survival, no differences were found between groups: all *p* values > 0.01 when considering both risk alleles individually and combinations of alleles or polygenic risk assessment [14].

CONCLUSION

The multifaceted nature of the risk of COVID-19 course severity requires consideration of multiple factors: clinical and demographic parameters, comorbid background, concomitant therapy, patient genetics, etc. Within the current understanding of COVID-19's nature, assessing the contribution of a patient's genetic profile to the severity of the course and clinical outcomes of the disease remains challenging. The problem requires further study.

Conflict of interest

The authors declare the absence of a conflict of interest.

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Information about the authors

Sherzod P. Abdullaev – Cand. Sc. (Biol.), Head of the Department of Predictive and Prognostic Biomarkers, Research Institute of Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: abdullaevsp@gmail.com, https://orcid.org/0000-0001-9001-1499

Natalia P. Denisenko – Cand. Sc. (Med.), Deputy Director, Research Institute of Molecular and Personalized Medicine, Associate Professor at the Department of Clinical Pharmacology and Therapy named after Academician B.E. Votchal, Russian Medical Academy of Continuous Professional Education, e-mail: natalypilipenko3990@gmail.com, https://orcid.org/0000-0003-3278-5941

Ilyas I. Temirbulatov – Postgraduate at the Department of Clinical Pharmacology and Therapy named after Academician B.E. Votchal, Russian Medical Academy of Continuous Professional Education, e-mail: temirbulatov.ilyas@gmail.com, https://orcid.org/0000-0002-1242-0833

Anastasia A. Kachanova – Junior Research Officer at the Department of Predictive and Prognostic Biomarkers, Research Institute of Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: aakachanova@yandex.ru, https://orcid.org/0000-0003-3194-4410

Svetlana N. Tuchkova – Junior Research Officer at the Department of Predictive and Prognostic Biomarkers, Research Institute of Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: svetlanatuch1998@gmail.com

Elizaveta V. Mikhaylenko – Postgraduate at the Department of Pharmacology I.M. Sechenov First Moscow State Medical University (Sechenov University), e-mail: elizavetamikhaylenko@gmail.com, https://orcid.org/0000-0002-6175-8179

Alexander V. Kryukov – Cand. Sc. (Med.), Associate Professor at the Department of Clinical Pharmacology and Therapy named after Academician B.E. Votchal, Russian Medical Academy of Continuous Professional Education, e-mail: alex.kryukov90@yandex.ru, https://orcid.org/0000-0001-7903-2977

Timur T. Valiev – Dr. Sc. (Med.), Docent, Associate Professor at the Department of Pediatric Oncology named after Academician L.A. Durnov, Russian Medical Academy of Continuous Professional Education, e-mail: timurvaliev@mail.ru, https://orcid.org/0000-0002-1469-2365

Karin B. Mirzaev – Dr. Sc. (Med.), Vice-Rector for Research and Innovation, Director, Research Institute of Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: karin05doc@yandex.ru, https://orcid.org/0000-0002-9307-4994

Dmitry A. Sychev – Dr. Sc. (Med.), Professor, Member of the RAS, Rector, Head of the Department of Clinical Pharmacology and Therapy named after Academician B.E. Votchal, Russian Medical Academy of Continuous Professional Education, e-mail: dimasychev@mail.ru, https://orcid.org/0000-0002-4496-3680

INFLUENCE OF ALPHA-GLUTAMIL-TRYPTOPHAN ON THE BACKGROUND AND INDUCED ACTIVITY OF FACTORS OF ADAPTIVE IMMUNITY FOR PREVENTION

Ruleva A.A. [1],
Krasnov A.A. [2],
Petlenko S.V. [3],
Zaplutanov V.A. [4],
Apryatina V.A. [5]

[1] Pediatric Research and Clinical Center for Infectious Diseases, Federal Medical Biological Agency (Professora Popova str. 9, Saint Petersburg 197022, Russian Federation)
[2] Herzen State Pedagogical University (Embankment of the Moika River 48, Saint Petersburg 191186, Russian Federation)
[3] Golikov Research Center of Toxicology (Bekhtereva str. 1, Saint-Petersburg 192019, Russian Federation)
[4] St. Petersburg Institute of Bioregulation and Gerontology (Dinamo Ave. 3, Saint Petersburg 197110, Russian Federation)
[5] Institute of Translational Biomedicine, St. Petersburg State University (Universitetskaya emb. 7–9, Saint Petersburg 199034, Russian Federation)

Corresponding author:
Anna A. Ruleva,
e-mail: Ruleanna@yandex.ru

ABSTRACT

Background. *During the ongoing COVID-19 pandemic and in the season of rising incidence of other respiratory infections, it is relevant to use preventive measures of non-specific prophylaxis. Synthetic peptides are widely considered as a tool. The representative of this group is the synthetic analogue of thymus regulatory peptides Thymogen, which has been used in Russia for more than 20 years in the treatment of acute and chronic infection diseases.*

The aim of the study. *To evaluate the effect of Thymogen, a dosed nasal spray, on induced parameters of the immune system during prophylactic use in healthy volunteers.*

Materials and methods. *Twenty healthy volunteers received Thymogen nasal dosed spray (OOO Cytomed, Russia) at a dose of 25 μg twice a day for 10 days. A comparative assessment of immunological parameters was carried out in dynamics: before the start of therapy, on days 6 and 11 of taking the drug and 14 days after the end of the course. Clinical observation was carried out from day 1 to day 11, registration of adverse events – the entire period of the study for 24 days.*

Results. *A statistically significant increase in virus-induced α-interferon production by blood cell culture on day 11 of Thymogen administration was revealed. This effect persisted for another 14 days after the end of the course. No statistically significant differences in the dynamics of bactericidal and phagocytic activity of neutrophils, serum α- and γ-interferon were observed.*

Conclusion. *The use of the Thymogen spray preparation at a dose of 25 μg for 10 days was considered safe, did not affect the morphofunctional state of the immune system, but promoted a statistically significant increase in the production of α-interferon in response to the inducing effect of the in vitro viral pathogen. As a result, the preparation can be recommended for prophylactic use during the period of high incidence in acute respiratory infections.*

Key words: *alfa-glutamyl-tryptophan, interferon-alpha, viral infections, healthy volunteers, non-specific prophylaxis, factors of innate immunity*

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ВЛИЯНИЕ АЛЬФА-ГЛУТАМИЛ-ТРИПТОФАНА НА ФОНОВУЮ И ИНДУЦИРОВАННУЮ АКТИВНОСТЬ ФАКТОРОВ ВРОЖДЁННОГО ИММУНИТЕТА ПРИ ПРОФИЛАКТИЧЕСКОМ ПРИМЕНЕНИИ

Рулева А.А. [1],
Краснов А.А. [2],
Петленко С.В. [3],
Заплутанов В.А. [4],
Апрятина В.А. [5]

[1] ФГБУ Детский научно-клинический центр инфекционных болезней ФМБА России (197022, г. Санкт-Петербург, ул. Профессора Попова, 9, Россия)
[2] ФГБОУ ВО «Российский государственный педагогический университет им. А.И. Герцена» (191186, г. Санкт-Петербург, набережная реки Мойки, 48, Россия)
[3] ФГБУ «Научно-клинический центр токсикологии имени академика С.Н. Голикова Федерального медико-биологического агентства» (192019, г. Санкт-Петербург, ул. Бехтерева, 1, Россия)
[4] АННО ВО НИЦ «Санкт-Петербургский институт биорегуляции и геронтологии» (197110, г. Санкт-Петербург, просп. Динамо, 3, Россия)
[5] Институт трансляционной биомедицины, ФГБОУ ВО «Санкт-Петербургский государственный университет» (199034, г. Санкт-Петербург, Университетская наб., 7–9, Россия)

Автор, ответственный за переписку:
Рулева Анна Александровна,
e-mail: Ruleanna@yandex.ru

РЕЗЮМЕ

В период продолжающейся пандемии COVID-19 и в сезон подъёма заболеваемости другими респираторными инфекциями остаётся актуальным использование неспецифической профилактики. В этой связи в качестве инструмента широко применяются синтетические пептиды. Представителем этой группы является синтетический аналог регуляторных пептидов вилочковой железы Тимоген, который применяется в России уже более 20 лет для лечения острых и хронических инфекционных заболеваний.

Цель исследования. *Оценить действие препарата Тимоген спрей на индуцированные показатели иммунной системы при профилактическом применении у здоровых добровольцев.*

Материалы и методы. *20 здоровых добровольцев получали препарат Тимоген спрей (АО «МБНПК «Цитомед», Россия) в дозе 25 мкг 2 раза в сутки в течение 10 дней. Иммунологические показатели оценивали в динамике: до начала терапии, на 6-й и 11-й дни приёма и через 14 дней после окончания курса. Клиническое наблюдение осуществляли с 1-го по 11-й дни, регистрацию нежелательных явлений – в течение всего периода исследования (24 дня).*

Результаты исследования. *Выявлено статистически значимое увеличение вирус-индуцированной продукции α-интерферона культурой клеток крови к 11-му дню приёма Тимогена. Этот эффект сохранялся ещё в течение 14 дней после окончания курса. Статистически значимых различий в динамике бактерицидной и фагоцитарной активности нейтрофилов, сывороточного α- и γ-интерферона не получено.*

Заключение. *Использование препарата Тимоген спрей в дозе 25 мкг в течение 10 дней было безопасным, не влияло на морфофункциональное состояние иммунной системы, но способствовало статистически значимому увеличению продукции α-интерферона в ответ на индуцирующее воздействие вирусного патогена in vitro. Это позволяет рекомендовать препарат для профилактического применения в период подъёма заболеваемости острыми респираторными инфекциями.*

Ключевые слова: *альфа-глутамил-триптофан, альфа-интерферон, вирусные инфекции, здоровые добровольцы, неспецифическая профилактика, факторы врожденного иммунитета*

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INTRODUCTION

Innate immunity is one of the most important factors determining the outcome of infection. Essentially all cells can participate in its realisation. The main structures responsible for innate immunity are monocytes, macrophages, dendritic cells (DCs), neutrophils and NK (natural killers) cells [1–4]. Innate immune cells carry receptors (PRR, pattern recognition receptors) such as Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), C-type lectin superfamily (CLSF), recognizing nonspecific structures of microorganisms (PAMP, pathogen-associated molecular patterns) [2, 3, 5]. After PRR binding to the ligand, intracellular biochemical cascades are triggered, leading to cellular activation. Activated cells initiate phagocytosis, secretion of reactive oxygen species and a number of cytokines (tumour necrosis factor α (TNF-α), interleukin (IL) 1β, IL-6, IFN (interferon) type 1), which in turn induce inflammation and other antiviral responses [2–4]. Phagocytosis is a multi-step process initiated by pathogen recognition that leads to pathogen uptake, phagosome maturation to eliminate the pathogen and then phagolysosome disintegration and pathogen inactivation [6].

The response of NK cells includes cytotoxicity and the release of cytokines (TNF-α and IFN-γ), which is closely related to the activation of receptors that activate NK cells and the blocking of inhibitory receptors on their surface [5]. The balance of these receptors' engagement protects normal cells from the harmful effects of NK cells while activating them to destroy virus-infected target cells [1, 5].

A key mechanism of innate immunity is the production of IFN type 1. It represents a highly optimised systemic response that provides a first line of defense against a wide range of viral infections. Failure to generate an effective IFN response against the virus leads to chronic infection, while excess IFN production leads to autoaggression [7]. Natural and recombinant interferons are among the most widely used biological therapeutic immunotropic agents in the world [8].

Currently, three major families of IFNs are known – types 1, 2 and 3. IFNs of type I (IFN-α/β) and 3 (IFN-λ) are primarily produced as the first line of defense and can be produced by most cell types, while IFNs of type 2 (IFN-γ) are mainly produced by secondary specialized immune subpopulations (e.g., NK cells or T cells) [2, 9]. Consequently, genetic determination of IFN type 1 and 3 synthesis is critical for controlling the susceptibility and course of viral infections. These IFNs are induced by a variety of systems including dozens of recognition receptors such as RIG-1, MDA-5 (melanoma differentiation-associated protein 5), TLR, RLR, STING (stimulator of interferon genes) [9]. It is also generally acknowledged that type 1 IFN production is induced by interaction with bacterial receptor ligands of innate immune cells. Furthermore, interferons activate higher-order processes (cellular activity, proliferation, differentiation and T cell function) that are critical for controlling viral infections. Deficiencies in the production or signalling of IFN-1 or IFN-3 nor in the antiviral proteins they induce are closely associated with the severe course of the disease [9].

Indeed, genetic deficiencies associated with the induction of IFN-α as well as IFN-neutralising autoantibodies have been identified in individuals with severe COVID-19 [10]. These correlations have epidemiologic significance. For example, 1.5 % of severe COVID-19 cases can be attributed to a specific TLR deficiency, while about 10 % of people with severe COVID-19 have autoantibodies against one or more type 1 or type 3 IFNs. In addition, ISG OAS1 allelic variants also predict the severity of COVID-19. These data definitively indicate that interferons are indispensable for the control of SARS-CoV-2 and prevention of severe COVID-19, as well as other respiratory viral infections (particularly influenza), where, due to the peculiarities of pathogens, disturbances in the system of immune response regulation are possible [11].

Thus, the innate immune response involves a coordinated chain of induced gene products, pre-formed immune effectors, biochemical signalling cascades and specialised cells [2, 12]. An important mechanism in the antiviral defense chain is the regulation of interferon production, and the degree of this induction allows predicting the course and outcome of infectious diseases, primarily of viral nature. Excessive activation of nocifensors can cause the development of pathological processes. A balance between pathogen defense and pathophysiological manifestations is important [13]. In fact, the presence of prolonged hyperinterferonemia during prophylactic use of interferon inducers (and some of this group of drugs can be used for up to 4 weeks or more) in the absence of a pathogen may contribute exclusively to the development of side effects, of which this group of cytokines (IFNs) has a huge number (from intestinal dysfunction and "flu-like state" to the development of "chronic fatigue and immune dysfunction syndrome" and dementia) [1, 14, 15]. Consequently, in our opinion, it is much more reasonable and effective, pathogenetically justified and safe to use drugs that do not induce but regulate the endogenous interferon synthesis in compliance with the needs and condition of the organism in order to prevent infectious diseases.

Synthetic peptides have been considered as a tool for regulation and activation of the interferon system by a number of studies. Key transcriptional peptide factors involved in the regulation of the immune response, such as NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), JAK-STAT (Janus kinase – signal transducer and activator of transcription) and IRFs (interferon regulatory factors), were originally discovered in bony fishes. The critical regulators of type 1 IFNs are IRF3 and IRF7, while IRF1, IRF5 and IRF8 trigger IFN responses in a cell-specific manner [16]. In the work of R. Pandey et al. [17], devoted to the development of immunoprophylaxis methods for visceral leishmaniasis, it was revealed that individual synthetic peptides or their mixtures significantly activated IFN-γ secretion. This was confirmed by an increase in intracellular cytokines with a significant increase in IFN-γ produced by $CD4^+$ T cells.

Since 2003, a synthetic dipeptide identical to the natural compound isolated by chromatographic method from thymus extract, the drug Thymogen, has been registered and used in Russia [18]. One of the main points underlying the present study was to investigate the immunologic mechanisms underlying the prophylactic effect of the preparation against acute and chronic viral and bacterial diseases of the upper respiratory tract in healthy volunteers.

In this academic research work, the key objective was to evaluate the effect of Thymogen in relation to baseline and induced immune system parameters during prophylactic use in healthy volunteers. Bactericidal and phagocytic activity of neutrophil granulocytes, spontaneous and virus-induced production of IFN-α by peripheral blood cells, and the content of IFN-α and IFN-γ in serum were studied. We also analysed the safety and tolerability of the studied drug in healthy volunteers during its prophylactic administration for 10 days. The terms of the study – from June 04, 2021, to July 05, 2021.

MATERIALS AND METHODS

A total of 20 healthy male volunteers aged between 18 and 40 years participated in the study. The inclusion criteria were absence of acute and exacerbation of chronic diseases at least 1 month before inclusion in the study; any vaccination at least 6 months before the commencement of the study.

The study preparation was Thymogen, nasal dosed spray (manufacturer — Medical and Biological Research and Production Complex OOO Cytomed, Russia). The active substance is alpha-glutamyl-tryptophan (Thymogen sodium in terms of Thymogen) 25 μg/dose. Excipients: sodium chloride 900 μg; benzalkonium chloride 10 mcg; water, purified to 0.1 ml.

The study was approved by the independent ethical committee 'BioEthics' (Protocol No. 156 dated May 27, 2021), Saint Petersburg, Russia.

All subjects included in the study received Thymogen spray according to a single regimen at a dose of 25 μg (1 injection) into each nasal passage 2 times a day in the morning and evening for 10 days. After completion of the course of preparation administration, the volunteers were monitored for another 14 days. Complaints, life and medical history, data related to the use of medications, and physical examination were collected before commencement of drug administration as well as at each medical surveillance.

Physical examination included measurement of vital signs: systolic (SBP) and diastolic (DBP) blood pressure, heart rate (HR), respiratory rate (RR), body temperature (BT). To measure vital signs, we used certified instruments (ASG, Japan; Armed YX200, Russia) designed for use in clinical trials. The total drug administration and observation period for each volunteer was 24 days and included 4 visits (day 1 – before drug administration; day 6, day 11; day 24), during which a complete physical examination and blood collection for laboratory diagnosis were performed.

Immunological examination included determination of bactericidal activity of peripheral blood neutrophil granulocytes (according to the NBT-test (nitroblue tetrazolium reduction test) spontaneous and induced by zymosan) by spectrometry on a spectrophotometer (Infinite F50; Austria); assessment of phagocytic activity of peripheral blood neutrophil granulocytes (percent of neutrophils that absorbed yeast; phagocytic index; completion of phagocytosis) by light microscopy (Leica DM LS2; Leica, USA); assessment of spontaneous and induced IFN-α production and the content of IFN-α and IFN-γ in serum by immunoenzyme method (commercial kits of OOO Vector Best, Russia). Spontaneous and Newcastle disease virus-induced IFN-α production was determined in supernatants of daily whole blood culture. The bactericidal stimulation index was calculated as the ratio of induced bactericidality to spontaneous bactericidality; the phagocytosis index was calculated as the average number of yeasts absorbed by one phagocyte. Phagocytosis completion was calculated as the ratio of phagocytic indices in samples with and without the addition of fetal serum.

Safety and tolerability of Thymogen was assessed by the frequency of adverse events (AEs) and serious adverse events (SAEs) according to the tolerability grading: "good" – absence of AEs; "satisfactory" – presence of mild AEs not requiring medication correction of the condition; "unsatisfactory" – presence of several AEs requiring medication treatment. Also within the framework of safety assessment we studied the dynamics of vital signs: SBP and DBP, HR, RR, BT. Tolerability was assessed based on subjective assessment by volunteers and by maintaining adherence to the study drug regimen (compliance).

Statistical analysis

Statistical data processing was performed using specialized software Statgraphics Centurion 18, version 18.1.12 (Statgraphics Technologies, Inc., USA) and RStudio, version 1.1.442 (RStudio PBC, USA), Rmisc packages (version 1.5), psych (version 2.1.6), nortest (version 1.0-4). To test the normality of the compared samples, the Lilliefors test was applied with a critical value of $p = 0.2$. Comparisons between related groups were performed using one-way ANOVA with repeated measures. The sphericity of the data was analyzed using Mauchly's test with a statistical significance level of 95 %. If the distribution of the parameter in the group deviated from normal, the nonparametric Friedman test was applied. The obtained data are presented as mean (M), standard deviation (σ), standard error of the mean (SE) or median (Me), quartile deviations (Q25%; Q75%) or interquartile range (IQR) (Q25%–Q75%), minimum (min) and maximum (max) values. The critical p value was considered to be 0,05.

In case differences between groups (at different time points) were revealed, posterior (post-hoc) tests for pairwise comparison were used to determine them: Fisher's

method after analysis of variance, Bonferroni criterion after Friedman's test with adjustments for multiple comparisons (with a power of 95 %).

The object of research

The mean age of the volunteers included in the study was 28.25 ± 6.69 years, with a minimum age of 18 years and a maximum age of 40 years. The group was homogeneous in terms of demographics and anthropometrics. A full course of the drug was provided to 17 volunteers and an incomplete course to 3 of them. All 20 study subjects were included in the analyses.

RESULTS

Assessment of spontaneous and induced IFN-α production under the influence of the drug

When the spontaneous production of IFN-α by daily culture of cells isolated from the peripheral blood of volunteers was examined on day 1 (Me = 5 pg/ml; IQR = 2.75 pg/ml) before drug administration compared to the values on day 6 (Me = 5 pg/ml; IQR = 0 pg/ml) (p = 0.026), statistically significant differences were found; however, these values were similar and such differences are not clinically significant (Table 1).

When Newcastle disease virus-induced IFN-α production by whole blood cells was assessed, statistically significant differences in this parameter were observed on days 1 and 11, on days 1 and 24, and between days 6 and 24 (F = 31.7; p = 0.000000604317) (Fig. 1).

In the analysis of the study results, a statistically significant increase in induced IFN-α production by blood cells during prophylactic administration of Thymogen for a course of 10 days was observed by day 11 of follow-up (day 11: Me = 390.5 pg/ml, IQR = 290.75 pg/ml; day 1: Me = 194.5 pg/ml, IQR = 149.25 pg/ml; $p < 0.001$). Furthermore, a statistically significant ($p < 0.001$) increase in this index 14 days after the end of the course (day 24: Me = 550.5 pg/ml; IQR = 190.25 pg/ml) compared to day 1 (Me = 194.5 pg/ml; IQR = 149.25 pg/ml) and day 6 (Me = 223.5 pg/ml; IQR = 164.5 pg/ml) was also revealed (Fig. 1).

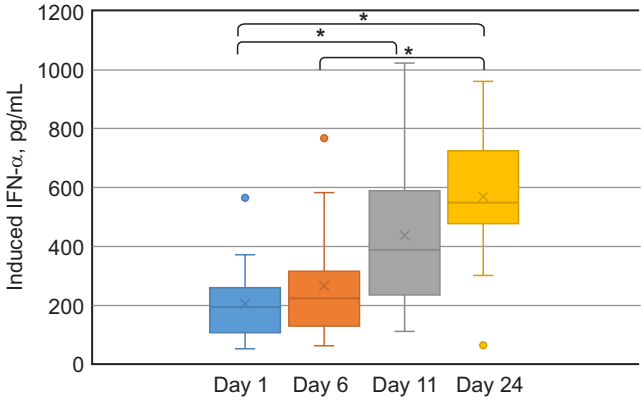

FIG. 1.

*Virus-induced production of IFN-α by daily culture of whole blood from volunteers in dynamics during 10-day administration of Thymogen spray (n = 20): * – statistically significant differences ($p < 0.001$), Friedman's nonparametric test; day 1, 6, 11 and 24 – control points of the study*

Accordingly, the present study reveals that virus-induced IFN-α production, reflecting one of the mechanisms of increasing the organism's immunity to viral pathogens, statistically significantly increases by the end of the drug administration (after 10 days) and continues to increase during 2 weeks of further follow-up. Consequently, prophylactic use of Thymogen in the spray form allows activating the ability of peripheral blood cells to produce IFN-α in response to viral pathogen exposure, and this effect persists for at least 14 days after the end of the course of the studied drug application.

Results of serum IFN-α and IFN-γ assessment

Comparison of IFN-α levels in the serum of healthy volunteers during prophylactic administration of Thymogen revealed statistically significant differences in this parameter on days 6, 11 and 24, despite the fact that the serum IFN-α levels were similar in absolute values (Me = 5 pg/ml; IQR = 0 pg/ml) compared to the values on day 1 (Me = 5 pg/ml; IQR = 2.25 pg/ml; p=0.0004), however, this change was not clinically significant (Table 1).

TABLE 1

SPONTANEOUS PRODUCTION OF IFN-α BY DAILY WHOLE BLOOD CULTURE

Indicators	Reference values	Control points	Me	Q25%	Q75%	min	max
IFN-α spontaneous, pg/ml	3–30	Day 1	5	5	7.75	5	19
		Day 6	5*	5	5	5	5
		Day 11	5	5	5	5	15
		Day 24	5	5	5	5	13

Note. * – statistically significant differences compared to the initial value ($p < 0.05$); Me – median; Q25%, Q75% – 25th and 75th quartiles, respectively; min – minimum; max – maximum.

Examination of IFN-γ in serum revealed no statistically significant differences in all controls ($p > 0.05$). The index did not change throughout the study (Me = 2 pg/ml; IQR = 0 pg/ml) (Table 2).

Results of the assessment of phagocytic and bactericidal activity

According to the results obtained, no statistically significant differences in the dynamics of such parameter as neutrophil bactericidal activity, including spontaneous and induced, were revealed in the present study. The indices had no statistically significant variations in the study controls (Table 3).

Similar results were obtained when studying the parameters of neutrophil phagocytic activity (Table 4), including neutrophil phagocytosis, phagocytosis completion and phagocytosis index.

The present study reveals that in healthy volunteers without immune system disorders and in the absence of acute diseases / conditions prophylactic use of Thymogen spray 2 times a day for 10 days does not affect the parameters of bactericidal activity of neutrophil granulocytes of peripheral blood, determined by NBT-test, and does not lead to changes in the phagocytic activity of neutrophils.

Analysis of the safety and tolerability

As part of the safety and tolerability analysis, vital signs were evaluated in dynamics: SBP, DBP, HR, RR and BT, which did not reveal statistically significant differences ($p > 0.05$) in healthy volunteers throughout the entire follow-up period (i.e. at the control points of the study – days 1, 6, 11 and 24) (Table 5).

In 11 (55 %) volunteers at different control points of the study, there were observed episodes of blood pressure rise, and in all cases the increase in SBP and / or DBP did not exceed 10 mmHg compared to the values taken as reference. The recorded abnormalities were classified by the authors as clinically insignificant, not requiring therapeutic measures and not being associated with the use of Thymogen.

No SAEs were observed during the study conducted. Among the recorded AEs, mucous nasal discharge ($n = 1$) and nasal congestion ($n = 1$) were observed on days 1–10 of the study, which resolved independently. Mucous discharge was observed in the mornings within 1 h after Thymogen administration and nasal congestion was observed 20 min after taking the drug within 3–4 h, which were considered by the authors to be drug-related conditions. The AEs were of mild severity. No clinical manifestations of allergic reactions, such as urticaria, rash, anaphylactic reactions, were observed during the use of the drug.

Thus, the obtained data about prophylactic use of Thymogen spray 2 times a day over a 10-day period indicate satisfactory tolerability of the drug, its safety and absence of adverse effects on the main vital signs.

DISCUSSION

According to the results obtained in the course of the study, a statistically significant increase in virus-induced IFN-α production by daily culture of peripheral blood cells was revealed after 10 days of Thymogen administration and continued growth of this indicator for 14 days after the end of the course. Consequently, prophylactic use of Thymogen spray increases the ability of peripheral blood cells to produce IFN-α in response to viral pathogen exposure during the period of drug administration and for at least 2 weeks afterwards. This mechanism of biologi-

TABLE 2
SERUM IFN-α AND IFN-γ LEVELS

Indicators	Reference values	Control points	Me	Q25%	Q75%	min	max
IFN-α serum, pg/ml	0–5	Day 1	5	2.75	5	1	5
		Day 6	5*	5	5	5	5
		Day 11	5*	5	5	5	5
		Day 24	5*	5	5	5	5
IFN-γ serum, pg/ml	0–5	Day 1	2	2	2	2	2
		Day 6	2	2	2	2	22
		Day 11	2	2	2	2	2
		Day 24	2	2	2	2	2

Note. * – statistically significant differences compared to the initial value ($p < 0.05$); Me – median; Q25%, Q75% – 25th and 75th quartiles, respectively; min – minimum; max – maximum.

TABLE 3

RESULTS OF ASSESSMENT OF NEUTROPHIL BACTERICIDAL ACTIVITY IN THE DYNAMICS OF THE STUDY

Indicators	Reference values	Control points	Me	Q25%	Q75%	min	max
Spontaneous neutrophil bactericidality, units/million cells	70–120	Day 1	98	74.25	112.5	68	116
		Day 6	93	85	104	62	124
		Day 11	92	87.25	102	71	124
		Day 24	88	82	98	68	116
Induced neutrophil bactericidality, units/million cells	150–200	Day 1	140	116	177.2	94	202
		Day 6	152	138	169.8	84	198
		Day 11	145	131.5	170.8	88	198
		Day 24	156.5	139.8	167.5	102	195
Stimulation index	1,2–2	Day 1	1.55	1.3	1.725	1.2	1.8
		Day 6	1.6	1.4	1.8	1.3	1.9
		Day 11	1.6	1.5	1.725	1.2	1.9
		Day 24	1.8	1.575	1.8	1.4	1.9

Note. Me – median; Q25%, Q75% – 25th and 75th quartiles, respectively; min – minimum; max – maximum.

TABLE 4

RESULTS OF ASSESSMENT OF NEUTROPHILS PHAGOCYTIC ACTIVITY IN THE DYNAMICS OF THE STUDY

Indicators	Reference values	Control points	Me	Q25%	Q75%	min	max
Neutrophil phagocytosis, %	65–88	Day 1	68	66	68	65	69
		Day 6	67.5	66	68	65	69
		Day 11	68	66	68	65	69
		Day 24	68	67	68	65	69
Completion of phagocytosis, coefficient	1–1,2	Day 1	1	1	1	0.9	1
		Day 6	1	1	1	0.9	1
		Day 11	1	1	1	0.9	1
		Day 24	1	1	1	1	1
Phagocytosis index	2,3–3	Day 1	2.3	2.3	2.4	2.2	2.8
		Day 6	2.3	2.3	2.4	2.2	2.7
		Day 11	2.3	2.3	2.4	2.2	2.6
		Day 24	2.3	2.3	2.4	2.2	2.5

Note. Me – median; Q25%, Q75% – 25th and 75th quartiles, respectively; min – minimum; max – maximum.

TABLE 5
DYNAMICS OF VITAL SIGNS DURING THE STUDY

Indicators	Reference values	Control points	M	σ	min	max
SBP, mmHg	110–135	Day 1	128.2	7.47	115	145
		Day 6	126.45	7.24	110	139
		Day 11	125.3	8.09	104	138
		Day 24	126.0	7.28	115	139
DBP, mmHg	60–85	Day 1	79.15	5.58	70	90
		Day 6	78.8	7.61	65	94
		Day 11	77.6	7.88	64	94
		Day 24	77.1	6.82	64	93
HR, bpm	60–90	Day 1	75.6	11.55	64	89
		Day 6	73.05	8.15	49	102
		Day 11	73.95	8.89	56	90
		Day 24	74.3	8.93	55	92
RR, respirations/min	up to 22	Day 1	16.05	0.94	14	18
		Day 6	16.2	1.06	14	18
		Day 11	15.9	0.85	14	18
		Day 24	16.4	1.05	14	18
BT, °C	35.5–36.9	Day 1	36.19	0.24	35.8	36.7
		Day 6	36.26	0.33	35.7	36.8
		Day 11	36.26	0.36	35.5	36.8
		Day 24	36.16	0.42	35.0	36.7

Note. M – mean value; σ – standard deviation; min – minimum; max – maximum.

cal activity should be considered effective in the prevention and treatment of respiratory viral infections of any etiology, and especially those whose immunopathogenesis is accompanied by inhibition of endogenous interferon synthesis, in particular COVID-19.

The study reveals that serum levels of IFN-α and IFN-γ remained at background levels throughout the study period.

The results obtained in healthy volunteers are consistent with the results of previous studies, in which it was revealed that Thymogen does not have the ability to stimulate a significant amount of endogenous interferon, which is associated with its mechanism of action, and is rather a regulator of this process, and to a greater extent against the background of primary antigenic induction [18].

Other authors note that Thymogen is an inducer of the production of endogenous interferon, mainly alpha and beta fractions. Specifically, in an *in vivo* study, Thymogen was administered subcutaneously to white mice and interferon levels in serum, lungs, and brain were assessed [19]. According to the results obtained, after already 2 hours the concentration of IFN in serum reached 10–20 IU/ml, persisted up to 4 h and decreased to zero a day after the drug administration.

Most likely, the different findings are related to the difference in the assessment time of endogenous interferon production – in the first day and in the distant period which are subject to significant changes in the normal course of the infectious process, as well as to the type of ob-

ject studied – animals or humans. In a number of drug study observations, the effects in animals often do not replicate the effects in humans.

Statistically significant changes in bactericidal activity of neutrophil granulocytes and phagocytic activity of neutrophils in healthy volunteers were not observed during the study, which suggests that in the absence of an infectious agent the drug does not change the background values, does not cause hyperactivation of protective factors of immunity, being activated only in the presence of a pathogen.

It should be noted that there are scientific studies confirming high local antiviral activity of Thymogen spray due to antiseptic action in suppressing the infectivity of SARS-CoV-2 virus [20] in patients with coronavirus infection. Besides, in earlier scientific studies devoted to examining the dynamics of immunological parameters in patients it was revealed that against the background of acute infectious diseases, i.e. in conditions of pre-formed pathogen exposure or other acute conditions, the use of Thymogen contributed to a faster recovery of reduced bactericidal activity of neutrophils determined by NBT-test. And this has been demonstrated for patients with severe mechanical trauma compared to patients receiving conventional treatment [21], as well as for patients with chronic pyoderma [22]. As concerns phagocytosis, previous studies have provided data about the stimulating effect of Thymogen in patients with chronic generalised periodontitis [23].

In the course of the study, it was also revealed that prophylactic use of Thymogen in the form of spray 2 times a day over a period of 10 days had no significant effect on the main vital signs of the organism. The diagnosed AEs were of mild severity and resolved independently. No allergic reactions to the administration of the drug were observed. Neither were there any episodes of study drug withdrawal initiated by the surveyors and study subjects. The data obtained confirm good tolerability and safety of Thymogen during its prophylactic use.

CONCLUSION

A statistically significant increase in virus-induced INF-α production by daily culture of peripheral blood cells in healthy volunteers was revealed by the study. The observed effect of the drug Thymogen, nasal dosed spray was persisted during the whole period of its administration – 10 days, and 14 days after the completion of the course. No statistically significant changes in neutrophil granulocyte bactericidal activity (including spontaneous and induced bactericidality) and neutrophil phagocytic activity (including neutrophil phagocytosis, phagocytosis completion and phagocytosis index) were observed during the study. The obtained data about the ability of the drug Thymogen to activate the reactions of innate immunity, involving the IFN system, deserve attention and further study of the mechanisms of its action.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Anna A. Ruleva – Cand. Sc. (Med.), Research Officer at the Department of Vaccinal Prevention and Postvaccinal Pathology, Pediatric Research and Clinical Center for Infectious Diseases, Federal Medical Biological Agency, e-mail: ruleanna@yandex.ru, https://orcid.org/0000-0002-5888-6313

Alexey A. Krasnov – Dr. Sc. (Med.), Associate Professor at the Department of the Medical Valeological Subjects, Herzen State Pedagogical University, e-mail: dr.krasnov_28@mail.ru, https://orcid.org/0000-0002-8732-6390

Sergey V. Petlenko – Dr. Sc. (Med.), Leading Research Officer, Golikov Research Center of Toxicology, e-mail: petlenko@mail.ru, https://orcid.org/0000-0002-2752-4598

Vasiliy A. Zaplutanov – Research Officer at the Laboratory of Peptide Chemistry of the Department of Biological Gerontology, St. Petersburg Institute of Bioregulation and Gerontology, e-mail: vasiliy@zaplutanov.ru, https://orcid.org/0000-0001-5294-6533

Vera A. Apryatina – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Neurobiology and Molecular Pharmacology, Institute of Translational Biomedicine, St. Petersburg State University, e-mail: vera1577@mail.ru, https://orcid.org/0000-0002-9819-6835

FLU IN CHILDREN: CLINICAL, LABORATORY INDICATORS AND CYTOKINE PROFILE PARAMETERS

Kazantseva E.D.,
Petrova A.G.,
Darenskaya M.A.,
Moskaleva E.V.,
Rychkova L.V.,
Semenova N.V.

Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)

Corresponding author:
Ekaterina D. Kazantseva,
e-mail: kat.smile7@yandex.ru

ABSTRACT

Introduction. *Respiratory diseases occupy a leading place in the structure of pathology of childhood. The proportion of influenza and acute respiratory viral infections among all infectious diseases is 90 %. The literature describes gender differences in the immune response to respiratory infections in children, but there is a gap in the description of the cytokine profile in children with influenza depending on gender and age.*

The aim of the study. *To analyze clinical and laboratory parameters as well as cytokine profile parameters in children with influenza.*

Materials and methods. *A single-stage descriptive study was conducted with the participation of 50 children from 1 to 11 years of age with a diagnosis of influenza who were on inpatient treatment at the Irkutsk Regional Infectious Diseases Clinical Hospital from December 2018 to January 2019. The clinical and laboratory features of the course of influenza in children and the duration of treatment were determined. The concentration of cytokines interleukin (IL) 1β, IL-4, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), interferon alpha and gamma (INF-α, INF-γ) in blood plasma was determined by enzyme-linked immunosorbent assay (ELISA) using diagnostic test systems manufactured by Vector-Best (Novosibirsk, Russian Federation) on the analyzer Multiscan EX (Thermo Electron, Germany). The control group consisted of practically healthy children without signs of acute respiratory viral infection (n = 50; mean age – 5.3 ± 2.6 years).*

Results. *When comparing clinical and laboratory data and cytokine profile parameters in children with influenza, no gender differences were found. There was a statistically significant increase in the level of pro-inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, INF-α, as well as CRP, anti-inflammatory cytokine IL-4 in influenza in all age categories, in contrast to the control group ($p < 0.05$).*

Conclusion. *Influenza in children of different sexes proceeds classically without a statistical difference in clinical and laboratory parameters and in the level of cytokines.*

Key words: *flu, clinic, cytokines, children*

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ГРИПП У ДЕТЕЙ: КЛИНИЧЕСКИЕ, ЛАБОРАТОРНЫЕ ПОКАЗАТЕЛИ И ПАРАМЕТРЫ ЦИТОКИНОВОГО ПРОФИЛЯ

Казанцева Е.Д.,
Петрова А.Г.,
Даренская М.А.,
Москалева Е.В.,
Рычкова Л.В.,
Семёнова Н.В.

ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)

Автор, ответственный за переписку:
Казанцева Екатерина Дмитриевна,
e-mail: kat.smile7@yandex.ru

РЕЗЮМЕ

Введение. *Болезни органов дыхания занимают ведущее место в структуре патологии детского возраста. Удельный вес гриппа и острой респираторной вирусной инфекции (ОРВИ) среди всех инфекционных болезней составляет 90 %. В литературе описаны гендерные различия в иммунном ответе при респираторных инфекциях у детей, однако существует пробел в описании цитокинового профиля у детей с гриппом.*

Цель исследования. *Провести анализ клинических и лабораторных показателей, а также параметров цитокинового профиля у детей с гриппом.*

Материал и методы. *Проведено одномоментное сравнительное исследование с участием 50 детей от 1 года до 11 лет с диагнозом грипп, которые находились на стационарном лечении в ОГБУЗ «Иркутская областная инфекционная клиническая больница» с декабря 2018 по январь 2019 г. Определялись клинические, лабораторные особенности течения гриппа у детей, продолжительность лечения. Концентрация цитокинов интерлейкина (IL) 1β, IL-4, IL-6, IL-8, фактора некроза опухоли α (TNF-α, tumor necrosis factor α), интерферона (INF) α, INF-γ и высокочувствительного С-реактивного белка (СРБ) в плазме крови определялась методом твердофазного иммуноферментного анализа (ИФА) с использованием диагностических тест-систем производства «Вектор-Бест» (г. Новосибирск) на анализаторе Мультискан EX (Thermo Electron, Германия). Контрольную группу составляли практически здоровые дети без признаков ОРВИ (n = 50; средний возраст 5,3 ± 2,6 года).*

Результаты*. При сравнении клинико-лабораторных данных и параметров цитокинового профиля у детей с гриппом гендерных различий не выявлено. Отмечается статистически значимое повышение уровня провоспалительных цитокинов IL-1β, IL-6, IL-8, TNF-α, INF-α, а также СРБ, противовоспалительного цитокина IL-4 при гриппе во всех возрастных категориях, в отличие от контрольной группы (p < 0,05).*

Заключение. *Грипп у детей разного пола протекает классически без статистической разницы в клинико-лабораторных показателях и в уровне цитокинов.*

Ключевые слова*: грипп, клиническая картина, цитокины, дети*

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INTRODUCTION

Among the aetiologically diverse groups of respiratory viral infections, influenza is a more serious concern and remains an uncontrollable global infection that causes enormous socioeconomic damage. The World Health Organization estimates that influenza and respiratory diseases affect 100 million people (5 to 30 % of the world's population) each year. According to the Ministry of Health of the Russian Federation, economic losses from influenza and acute respiratory viral infection (ARVI) account for 86 % of all damage caused by infectious diseases. The magnitude of the damage caused by influenza and influenza-like infections to public health and the economy of any country can be compared with those for injuries, cardiovascular diseases and malignant neoplasms [1].

Markers of the severe course of pandemic influenza AH1N1pdm09 in adolescents and adults are elevated blood concentrations of cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL) 6 [2]. Similarly, in pandemic influenza complicated by pneumonia, albeit in childhood, there is an increased synthesis of anti-inflammatory mediators and decreased concentration of pro-inflammatory cytokines IL-1β and TNF-α [3]. The same markers are assigned a leading role in the development of cytokine storm in influenza patients [4, 5].

Higher plasma IL-6 and TNF-α concentrations in nasopharyngeal wash samples have been described in overweight children [6, 7]. These children also have higher morbidity and mortality in influenza and a high risk of developing secondary bacterial infections [8].

A similar pattern of pro-inflammatory cytokine secretion of TNF-α and IL-6 has been observed in influenza type A and B in children. In influenza caused by influenza A virus, cytokine output with a predominance of Th2 inflammation is observed; IL-4 output is particularly elevated, which is not observed in influenza B [9].

There is uneven agreement among different authors as to which type of influenza virus variant – seasonal or pandemic – is predominant in childhood. Some authors indicate that seasonal influenza more often causes illness in children under 2 years of age: the infection is particularly severe in children under 6 months of age; pandemic influenza, on the contrary, more often affects children of preschool age, occurs more often in children over 5 years of age [10]. Other authors have reported the opposite: the pandemic variant of influenza is statistically significantly more common in children of the first year of life, while the seasonal variant is more common in children of primary school age [11].

Influenza has moderate form in young children, and only 16 % of cases have a severe course of the disease. Among the symptoms, catarrhal inflammation of the upper respiratory tract and intoxication syndrome predominate [12].

In the disease pattern of influenza in children, acetonemic syndrome is observed in 26.3 % of cases, as well as isolated cases of neurotoxicosis and haemorrhagic syndrome [13, 14].

Nowadays, it has been proved that influenza type A, especially the virus subtype containing neuraminidase N2, is associated with a more severe course of infection, more often complicated by secondary bacterial infection [15, 16].

In respiratory viral infections, oxidative stress is actively involved in the mechanisms of sustaining homeostatic disturbances; antioxidant drugs such as vitamin C, N-acetylcysteine, quercetin, glutathione, fat-soluble vitamins and polyunsaturated fatty acids have proven themselves in clinical trials in influenza, pneumonia and other respiratory diseases [17, 18].

Male subjects have higher synthesis of pro-inflammatory cytokines such as IL-6 and TNF-α; female subjects have high synthesis of the anti-inflammatory cytokine IL-10. Gender differences in immune response have been attributed to hormonal factors. There is speculation that women's defense against infections is associated with the pro-inflammatory effects of estradiol, while men's susceptibility to infections is associated with immunosuppression as a consequence of testosterone effects, possibly involving specific receptors [19].

There is a significant difference between girls and boys in the concentration of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), neutrophils in inflammatory processes in children with pneumonia, pyelonephritis, bronchiolitis [20].

Recently, there is a limited number of studies devoted to the analysis of cytokine profile parameters in children with influenza; there is also insufficient information on gender differences in acute respiratory viral infections in children.

THE AIM OF THE STUDY

To analyse clinical and laboratory parameters and to assess cytokine profile parameters in children with influenza.

MATERIALS AND METHODS

A one-stage comparative study was conducted with the participation of 50 children aged 1 to 11 years old, who from December 2018 to January 2019 were admitted for inpatient treatment in the Irkutsk Regional Infectious Diseases Clinical Hospital with a diagnosis of influenza.

Distribution of children by age groups was performed according to the periodisation of childhood using the scheme of N.P. Gundobin: early childhood – from 1 to 3 years; preschool age, middle childhood or the 1st period of childhood – from 3 to 7 years; younger school age, older childhood or the 2nd period of childhood – girls from 7 to 11 years, boys from 7 to 12 years.

The clinical features of the influenza course in children were analysed with consideration of the disease duration, number of days spent in hospital, main and concomitant diagnoses, presence of complications, disease outcome,

and history of influenza vaccine prophylaxis. Disease pattern was assessed by complaints, occurrence, nature and duration of symptoms of rhinitis, cough, intoxication (lethargy, refusal to eat, nausea or vomiting at the peak of fever, general weakness, sleep disturbance) and fever (body temperature increase from 37.2 to 38.0 °C – subfebrile fever; from 38.1 to 39.0 °C – febrile fever; from 38.1 to 39.0 °C – febrile fever; 39.1 to 40.0 °C – pyretic fever), other clinical manifestations (rash, cramps, sore throat, headache, muscle pain), deviations of laboratory indicators of general and biochemical blood tests and general urinalysis on admission, volume and duration of infusion, antiviral and antibacterial drug therapy throughout the disease.

The concentration of highly sensitive CRP and cytokines IL-1β, IL-4, IL-6, IL-8, TNF-α, INF-α, INF-γ in blood serum was determined by solid-phase enzyme immunoassay (EIA) using diagnostic test systems produced by Vector-Best (Novosibirsk) on a MultiscanEX microplate photometer (Thermo Electron, Germany). Blood sampling to determine the level of CRP and cytokines was performed in the first 3 days of the disease at admission to the hospital. The main group consisted of children diagnosed with influenza (n = 50), control group – of 50 practically healthy children without signs of ARVI or 14 days after acute viral infection (mean age of children – 5.3 ± 2.6 years). The study protocol was approved by the Biomedical Ethics Committee of Scientific Centre for Family Health and Human Reproduction Problems (excerpt from the minutes of the meeting No. 8.4 dated November 02, 2018).

Analysis and statistical processing of information were performed using MS Excel (Microsoft Corp., USA) and Statistica 6.0 application software package (StatSoft Inc., USA). The confidence interval with significance level of 95 % (95 % CI) for frequencies and fractions was calculated using an online calculator at Vassar Stats: Website for Statistical Computation[1]. Qualitative characters are presented as absolute (n) and relative values (P), quantitative characters are presented with median (Me) plus 25th and 75th quartiles (Q25; Q75). In the analysis of qualitative characters, the relative frequency of the characteristic (prevalence) P and 95 % CI were assessed. Statistical significance of intergroup differences in qualitative characters was assessed using the χ^2 criterion: at $P_{abs.} < 10$ – with Yates correction, at $P_{abs.} < 5$ – using Fisher's two-sided exact test. Statistical significance of two unrelated groups was assessed by Mann – Whitney test (U-test). The critical level of statistical significance in testing statistical hypotheses about the existence of differences between groups (p) was considered to be 0.05.

RESULTS AND DISCUSSION

In the study group, the sex distribution was almost equal: the proportion of boys was 52 % (n = 26), girls – 48 % (n = 24). The mean age of the children was 3 ± 2.6 years.

The diagnosis of influenza was verified by polymerase chain reaction comprehensive diagnosis with typing of influenza A/B strains in 96 % of children (n = 48) in the study group. Influenza A serotype H1N1sw2009 predominated in the etiologic structure: it was observed in 76 % of cases (n = 38). Influenza AH3N2 was the second most frequently observed – 16 % (n = 8). Clinically, influenza A was diagnosed in 4 % of patients (n = 2), mixed viral infection influenza AH1N1 + respiratory syncytial mixtovirus infection in 2 % (n = 1), and influenza A H1N1 + bocavirus mixtovirus infection in 2 % (n = 1).

Along with the main diagnosis, concomitant pathology was also considered: acute intestinal infection (rota- and norovirus etiology) in 16 % of cases (n = 8), atopic dermatitis in 2 % (n = 1), hypochromic anaemia in 6 % (n = 3). Complications of the underlying disease were observed in 30 % of patients (n = 15): acetone vomiting syndrome or ketoacidosis syndrome in 12 % (n = 6), pneumonia in 16 % (n = 8), acute obstructive bronchitis in 2 % (n = 1).

The mean duration of children's admission to hospital was 5 ± 1.6 days. In analysing the vaccination history, it was found that influenza vaccination was performed in 16 % of cases (n = 8), 72 % of patients (n = 36) were not vaccinated against influenza, and 12 % of patients (n = 6) had no information about vaccination.

Clinical features of the influenza course in children were assessed by the presence of complaints and objective examination data. The prevalence of complaints and clinical manifestations in the study group were as follows: pharyngeal hyperemia – 96 % (n = 48), rhinorrhea – 66 % (n = 33), cough – 84 % (n = 42), sore throat and pain when swallowing – 6 % (n = 3), back pain abdominal and loose stools – 8 % (n = 4), intoxication syndrome – 38 % (n = 19), including fever – 92 % (n = 46), lethargy, drowsiness – 44 % (n = 22), weakness – 34 % (n = 17), vomiting – 28 % (n = 14), decreased appetite – 26 % (n = 13), headache – 8 % (n = 4), convulsions – 4 % (n = 2), ear pain and dizziness – in single cases, muscle and joint pain was not observed in anyone. In describing the nature and duration of respiratory symptoms, children diagnosed with influenza were observed to have the following predominant symptoms: mucous runny nose – 72 % (n = 36) with a duration of 5 ± 2.48 days; dry cough – 84 % (n = 42) and wet cough – 6 % (n = 3). The median fever was 38.5 ± 0.68° and the duration of fever was 2 ± 1.45 days.

The clinic of influenza in children corresponds to the forme pleine: mucous runny nose, dry cough, fever, and hyperemia of the pharynx predominate in 96 % of cases. No statistical differences were revealed cause-specific to the gender of the child in terms of both the prevalence of clinical manifestations (Table 1) and the duration of the main symptoms (Table 2).

No differences between the gender groups were also revealed while comparing the main haematological and biochemical parameters of blood and urine in children with influenza (Table 3).

In the epidemic season of 2009, according to the findings of S.A. Chavanina et al. (2011), among the peculiarities of seasonal influenza it was observed that in 25 % of cases

[1] http://vassarstats.net/

patients were being admitted to hospital on the 6–9th day from the onset of the disease, often against the background of already developed complications. The height of fever was higher in children with seasonal influenza AH1N1 (mean – 39.2 °C vs. 37.9 °C in pandemic influenza A H1N1sw2009); marked intoxication and haemorrhagic syndrome were statistically significantly more prevalent in seasonal influenza AH1N1 [21]. The analysis of haematological changes revealed a high incidence of leukocytosis in patients with influenza AH1N1 (33.3 %) [22], while in our study we obtained data revealing that leukocytosis in children with influenza was observed in 6 % of cases and accelerated ESR syndrome in 42 % of cases.

The duration of infusion, antiviral and antibiotic therapy for influenza in children of different sexes did not differ. All hospitalized children were discharged with recovery.

Determination of cytokine and high-sensitivity CRP concentrations in children with influenza during the first days of hospitalisation was compared with a group of healthy children standardised by the copy-pair method. The method is based on the selection for each observation unit of the group under study by one or more characteristics, in this case – by gender, age; this method of selection is expedient for studying rare phenomena. The levels of CRP and cytokine profile parameters studied in healthy children were within the reference range according to the test system manufacturer's instructions: CRP < 5 pg/ml; IL-1β < 11 pg/mL; IL-4 < 4 pg/mL; IL-6 < 10 pg/mL; IL-8 < 12 pg/mL; TNF-α < 6 pg/mL; INF-α < 11 pg/mL; INF-γ < 20 pg/mL. In the serum of children with influenza, the concentrations of IL-1β, IL-4, IL-6, IL-8, TNF-α, INF-γ and CRP were significantly higher than the upper limit of the range.

A number of literature sources provide age and gender differences in the levels of pro- and anti-inflammatory cytokines in children, which is important to consider when interpreting cytokine status parameters [2, 21, 23].

No statistically significant differences were revealed by analysing cytokine levels associated with influenza in children of different genders (Table 4).

TABLE 1

MAIN CLINICAL SYMPTOMS IN CHILDREN WITH INFLUENZA ACCORDING TO GENDER (*n* = 50)

Indices	Influenza A, boys (*n* = 26)	Influenza A, girls (*n* = 24)	*P* (Fisher's exact criterion)
Mucous rhinitis	19 (73 %)	17 (70.8 %)	0.554
Dry cough	17 (65.4 %)	19 (79.2 %)	0.222
Fever	15 (57.7 %)	22 (91.6 %)	0.539
Intoxication	10 (38.5 %)	9 (37.5 %)	0.588
Pharynx hyperemic	25 (96.2 %)	23 (95.8 %)	0.734
Drowsiness, lethargy	11 (42.3 %)	11 (45.8 %)	0.513
Weakness	9 (34.6 %)	8 (33.3 %)	0.581
Vomiting	6 (23.1 %)	7 (29.2 %)	0.433

Note. * – differences are statistically significant at $p < 0.05$.

TABLE 2

DURATION OF MAJOR CLINICAL PARAMETERS IN CHILDREN WITH INFLUENZA ACCORDING TO GENDER

Duration of symptoms (days)	Influenza A, boys (*n* = 26) Me (Q25; Q75)	Influenza A, girls (*n* = 24) Me (Q25; Q75)	*p* (U-test)
Rhinitis	5 (3; 6)	5 (1.5; 5)	0.302
Cough	5 (4; 6)	5 (4; 7)	0.380
Fever	2 (2; 4.25)	2 (1; 3)	0.482

Note. * – differences are statistically significant at $p < 0.05$.

TABLE 3

LABORATORY PARAMETERS IN CHILDREN WITH INFLUENZA ACCORDING TO GENDER

Indices	Influenza A, boys (n = 26) Me (Q25; Q75)	Influenza A, girls (n = 24) Me (Q25; Q75)	p (U-test)
Erythrocytes, $\times 10^{12}$	4.35 (4.06; 4.70)	4.36 (4.07; 4.79)	0.917
Hemoglobin, g/L	120 (113.00; 127.00)	120 (113.25; 127.00)	0.796
Platelets, $\times 10^9$	226 (191.75; 250.75)	226 (187.25; 252.25)	0.358
Leukocytes, $\times 10^9$	3.57 (2.73; 5.00)	3.59 (2.74; 4.93)	0.796
Stab neutrophils, $\times 10^9$	1.84 (0.98; 3.34)	1.84 (0.98; 3.16)	0.876
Segmented neutrophils, $\times 10^9$	0.84 (0; 2.27)	0.00 (0.00; 1.37)	0.515
Lymphocytes, $\times 10^9$	1.32 (1.08; 1.89)	1.32 (1.09; 1.88)	0.764
Monocytes, $\times 10^9$	0.43 (0.34; 3.00)	0.48 (0.34; 3.00)	0.287
Eosinophils, $\times 10^9$	0.03 (0.01; 0.06)	0.03 (0.01; 0.07)	0.392
ESR, mm/h	12 (5.00; 19.00)	12 (5.00; 19.00)	0.179
C-reactive protein, mg/L	17.10 (3.87; 18.30)	15.10 (5.57; 18.10)	0.926
Total protein, g/L	67.10 (60.25; 70.93)	67.60 (60.63; 70.98)	0.983
Glucose, mmol/L	4.02 (3.52; 4.58)	4.02 (3.54; 4.49)	0.664
AST, U/L	51.25 (38.15; 68.38)	51.25 (38.36; 67.53)	0.529
ALT, U/L	15.25 (10.95; 21.25)	15.25 (10.85; 20.95)	0.860
Creatinine, μmol/L	44.9 (41.12; 53.15)	45.35 (41.33; 53.25)	0.278
Urea, mmol/L	1.53 (0.00; 3.86)	1,53 (0.00; 3.79)	0.967
Amylase, U/L	0 (0.00; 34.30)	0 (0.00; 35.10)	0.332
Urine specific gravity, g/L	1020 (1013.75; 1020.00)	1020 (1015; 1020)	0.470
Ketones in urine, mg/dL	0 (0.00; 0.50)	0 (0.00; 0.50)	0.489

Note. ESR – erythrocyte sedimentation rate; AST – aspartate aminotransferase; ALT – alanine aminotransferase; * – differences are statistically significant at $p < 0.05$.

TABLE 4

CYTOKINE LEVELS IN CHILDREN WITH INFLUENZA CAUSE-SPECIFIC TO GENDER

Cytokine concentration (pg/mL)	Influenza A, boys (n = 26) Me (Q25; Q75)	Influenza A, girls (n = 24) Me (Q25; Q75)	p (U-test)
IL-1β	8.10 (3.25; 12.45)	8.30 (2.75; 21.80)	0.853
IL-4	3.80 (1.95; 6.60)	3.2 (1.92; 4.45)	0.593
IL-6	18.30 (13.42; 32.77)	22.50 (14.20; 41.82)	0.509
IL-8	157.55 (72.02; 233.17)	183.70 (102.85; 287.30)	0.361
TNF-α	2.25 (1.70; 4.03)	2.55 (1.30; 4.52)	0.961
IFN-α	27.35 (4.90; 54.80)	21.4 (13.75; 59.03)	0.838
IFN-γ	1.60 (0.85; 5.50)	1.2 (0.35; 3.17)	0.289

Note. * – differences are statistically significant at $p < 0.05$.

Analysis of cytokine and CRP levels in influenza among children of different ages revealed that children of primary school age have increased synthesis of IL-1β, IL-6, IL-8, IFN-α, in contrast to young children, but no statistically significant differences were revealed ($p > 0.05$) (Table 5).

Increased synthesis of IL-4, TNF-α, IFN-γ was observed in young children, in contrast to preschool and primary school children; no statistically significant differences were revealed ($p > 0.05$) (Table 5).

TABLE 5

CYTOKINE AND C-REACTIVE PROTEIN LEVELS IN CHILDREN WITH INFLUENZA CAUSE-SPECIFIC TO AGE

Indices	Influenza A, Me (Q25; Q75)	*p* (U-test)
IL-1β[1]	5.50 (12.89; 28.64)	0.614
IL-1β[2]	6.60 (9.29; 17.26)	0.159
IL-1β[3]	14.50 (23.85; 63.29)	0.299
IL-4[1]	3.60 (1.95; 4.33)	0.470
IL-4[2]	3.35 (2.70; 5.02)	0.792
IL-4[3]	3.45 (1.86; 4.94)	0.760
IL-6[1]	14.80 (28.59; 63.55)	0.626
IL-6[2]	18.90 (11.00; 20.43)	0.278
IL-6[3]	31.60 (44.40; 117.85)	0.215
IL-8[1]	119.65 (88.71; 197.13)	0.581
IL-8[2]	199.24 (70.69; 131.32)	0.121
IL-8[3]	247.80 (76.87; 204.04)	0.161
TNF-α[1]	3.15 (3.15; 7.00)	0.277
TNF-α[2]	1.75 (1.18; 2.20)	0.769
TNF-α[3]	2.70 (15.69; 41.66)	0.117
IFN-γ[1]	2.55 (2.93; 6.51)	0.284
IFN-γ[2]	1.20 (2.19; 4.07)	0.953
IFN-γ[3]	1.40 (2.75; 7.31)	0.451
IFN-α[1]	31.85 (17.04; 37.86)	0.338
IFN-α[2]	17.00 (43.39; 80.61)	0.364
IFN-α[3]	45.75 (45.96; 121.99)	0.230
CRP [1]	14.55 (5.615; 12.477)	0.322
CRP [2]	17.20 (5.84; 10.85)	0.953
CRP [3]	15.25 (5.95; 15.80)	0.439

Note. [1] – age category of children from 1 year to 2 years 11 months 29 days ($n = 14$); [2] – age category of children from 3 years to 6 years 11 months 29 days ($n = 22$); [3] – age category of children from 7 to 11 years inclusive ($n = 14$); * – differences are statistically significant at $p < 0.05$.

Preschool children have increased synthesis of CRP, as opposed to children of other age categories; no statistically significant differences were revealed ($p > 0.05$) (Table 5). The pro-inflammatory cytokines IL-1β, IL-6, IL-8 and anti-inflammatory IFN-α are elevated in serum among children with seasonal influenza compared to healthy controls, with IL-6 concentration statistically significantly increased in all age groups ($p < 0.05$). In the age group of 7–11 years, a more significant increase was revealed in serum IL-1β – 14.50 (23.85; 63.29) pg/ml, IL-8 – 247 (76.87; 204.04) pg/ml, IFN-α – 45.75 (45.96; 121.99) pg/ml, compared to children of early and preschool age. Such results in school-age children can probably be explained both by a more efficient realisation of phagocytic neutrophil functions and by a more pronounced activity of inflammation. An increase in serum interleukins 1-β, IL-8 and INF-α is considered by some authors as unfavourable prognostic signs against the development of pneumonia and its prolonged and complicated course [21, 24, 25]. The interpretation of the increase in pro-inflammatory cytokine levels, according to the literature data, has a contradictory character and further requires the analysis of the relationship of systemic concentrations of IL-1β, IL-6, IL-8 and TNF-α with the severity and duration of intoxication syndrome, the peculiarities of the influenza course.

One of the key cytokines in the implementation of antiviral and anti-infectious immunity is IL-4, which provides induction of immune response through the humoral pathway. The cell-mediated Th1-type response is most effective in viral infections; therefore, in a number of diseases, IL-4 hyperproduction against the background of IFN-γ decrease is considered as an unfavourable prognostic sign [26]. However, there is a study that revealed increased levels of the anti-inflammatory cytokine IL-4 to be prognostically favourable in the course of pneumonia in preterm neonates, in contrast, an increase in TNF-α was associated with an unfavourable outcome of the disease [27]. Among children with influenza, a statistically significant increase in IL-4 was revealed in all age groups, in contrast to the control group ($p < 0.05$). The IFN-γ level during influenza in children of the 2nd and 3rd age groups was higher than in healthy children, and at early age had no statistically significant differences in the influenza acute period, which can be considered a failure of the interferon system in children under 3 years of age and can be regarded as a decrease in antiviral defense and as a predictor of a complicated, prolonged course of viral infection in this age group.

The analysis of CRP level as a marker of the acute phase of inflammation is of great diagnostic significance in bacterial infections, while in viral diseases the increase of CRP is determined less frequently, and a significant increase in serum CRP in influenza may be a prognostically unfavourable sign against the development of bacterial complications.

We have studied the relationship between the parameters of cytokine status and CRP in children with influenza as a cause-specific complication of pneumonia

(Table 6). Consequently, in children with uncomplicated influenza pneumonia a positive correlation between CRP and IL-1β levels was revealed, which is evidence of inflammation activity and timely activation of innate immunity factors. The negative correlation between CRP and IFN-γ levels in children with influenza without pneumonia is evidence of insufficiency of the interferon system in childhood during the acute phase of viral infection.

Influenza with pneumonia in children was characterised by a statistically significant direct correlation between CRP and IL-4 levels, which indicates deviation of Th0-lymphocyte differentiation by $CD4^+$/Th2-type and is a determining factor affecting the severity of clinical manifestations and outcome of influenza infection [28, 29].

CONCLUSION

Clinical manifestations of influenza in children aged 1 to 11 years, admitted to an infectious diseases hospital, correspond to the classic pattern/forme pleine: mucous runny nose, dry cough, fever, and pharyngeal hyperaemia predominate in 96 % of cases. Complications of the underlying disease were observed in 30 % of patients ($n = 15$) in the form of acetonemic vomiting syndrome or ketoacidosis syndrome, pneumonia, obstructive syndrome. No statistically significant differences were revealed in children with influenza cause-specific to gender, prevalence of clinical manifestations, duration of main symptoms, duration and intensity of infusion, antiviral and antibacterial therapy. The results of the analysis of islet-inflammatory, general clinical, biochemical indices of blood, urine, parameters of cytokine profile in children with influenza also revealed no statistically significant differences.

The study established the peculiarities of cytokine status in children during the acute phase of influenza. The increased concentration of CRP, systemic levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and anti-inflammatory IFN-α in influenza in children in all age groups, especially at the age of 7–11 years, requires further additional analysis and identification of the relationship of IL-1β, IL-6, IL-8 and TNF-α levels with the features of the disease pattern, in particular, with the severity of intoxication syndrome, in order to determine the degree of influence of each cytokine on the course and outcome of influenza in childhood.

The absence of statistically significant differences in IFN-γ levels at an early age during the acute period of viral infection compared with healthy children is alarming considering the risk of prolonged and complicated course of influenza, especially if the decrease in IFN-γ is accompanied by increased IL-4 levels. Increased systemic IL-4 level along with high concentration of CRP can be considered statistically significant signs of influenza complicated by pneumonia in childhood.

The revealed peculiarities of cytokine status in influenza among children allow to make some approximation to the understanding of the nature of the infectious process. Studies in this area are worth continuing, as they will have practical significance: identification of markers of adverse influenza course, development of a personalized approach to the treatment of acute respiratory infections in children under 3 years of age and timely prescription of medicinal products based on interferon and its inducers for prophylactic and therapeutic purposes, as well as the development of immunorehabilitation programmes.

Conflict of interest
The authors of this article declare no conflicts of interest.

TABLE 6

CORRELATION BETWEEN C-REACTIVE PROTEIN AND CYTOKINE LEVELS IN CHILDREN WITH INFLUENZA CAUSED-SPECIFIC TO THE PRESENCE OF PNEUMONIA COMPLICATION

Cytokines	Children with influenza without complications ($n = 42$)		Children with influenza complicated by pneumonia ($n = 8$)	
	r	*p*	*r*	*p*
IL-1β	0.309	0.008*	–0.142	0.735
IL-4	0.094	0.257	0.754	0.031*
IL-6	0.206	0.155	0.261	0.530
IL-8	0.217	0.123	0.428	0.289
TNF-α	0.265	0.088	0.144	0.732
IFN-α	–0.066	0.284	–0.595	0.119
IFN-γ	–0.477	0.001*	–0.239	0.567

Note. *r* – Spearman correlation coefficient; *p* – statistical significance of differences; * – differences are statistically significant at $p < 0.05$.

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Information about the authors

Ekaterina D. Kazantseva – Pediatrician, Postgraduate, Laboratory Assistant at the Laboratory of Infectology and Immunoprophylaxis in Pediatrics, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: kat.smile7@yandex.ru, https://orcid.org/0000-0003-0692-2295

Alla G. Petrova – Dr. Sc. (Med.), Professor, Head of the Laboratory of Infectology and Immunoprophylaxis in Pediatrics, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: rudial75@gmail.com, https://orcid.org/0000-0002-7965-8061

Marina A. Darenskaya – Dr. Sc. (Biol.), Chief Research Officer, Professor of the RAS, Head of the Laboratory of Pathophysiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: marina_darenskaya@inbox.ru, https://orcid.org/0000-0003-3255-2013

Ekaterina V. Moskaleva – Cand. Sc. (Med.), Research Officer at the Laboratory of Infectology and Immunoprophylaxis in Pediatrics, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: mkaterina@mail.ru, https://orcid.org/0000-0003-4196-0713

Lyubov V. Rychkova – Dr. Sc. (Med.), Professor, Corresponding Member of the RAS, Director, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-0117-2563

Natalya V. Semenova – Dr. Sc. (Biol.), Deputy Director for Science, Chief Research Officer at the Laboratory of Pathophysiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: natkor_84@mail.ru, https://orcid.org/0000-0002-6512-1335

CARDIOLOGY

FEATURES OF DRUG-DRUG INTERACTIONS RIVAROXABAN AND CALCIUM CHANNEL BLOCKERS DEPENDING ON THE *ABCB1* GENOTYPE (rs1045642 AND rs4148738) IN PATIENTS 80 YEARS OF AGE AND OLDER WITH NON-VALVULAR ATRIAL FIBRILLATION

Sychev D.A. [1],
Mirzaev K.B. [1],
Cherniaeva M.S. [2, 3],
Shakhgildyan N.V. [4],
Abdullaev Sh.P. [1],
Denisenko N.P. [1],
Sozaeva Zh.A. [1],
Kachanova A.A. [1],
Shastina V.R. [3, 5],
Gorbatenkova S.V. [3]

[1] Russian Medical Academy of Continuous Professional Education (Barrikadnaya str. 2/1, building 1, Moscow 125993, Russian Federation)
[2] Central State Medical Academy of the Administrative Directorate of the President of the Russian Federation (Marshala Timoshenko str. 19, building 1A, Moscow 121359, Russian Federation)
[3] War Veterans Hospital No. 2 of the Department of Health Services of Moscow (Volgogradskiy Ave. 168, Moscow 109472, Russian Federation)
[4] Lomonosov Moscow State University (Leninskie Gory str. 1, Moscow 119991, Russian Federation)
[5] I.M. Sechenov First Moscow State Medical University (Sechenov University) (Trubetskaya str. 8, building 2, Moscow 119991, Russian Federation)

Corresponding author:
Marina S. Cherniaeva,
e-mail: doctor@cherniaeva.ru

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ABSTRACT

Background. *The use of P-glycoprotein (P-gp) inhibitors and carriage of certain ABCB1 polymorphisms can lead to increased concentrations of rivaroxaban and the development of bleeding.*
The aim. *To study the features of drug-drug interactions (DDI) of rivaroxaban in patients over 80 years of age with non-valvular atrial fibrillation depending on the ABCB1 genotype (rs1045642 and rs4148738) using the example of verapamil (P-gp inhibitor) and amlodipine.*
Materials and methods. *One hundred and twenty-eight patients were examined (median age – 87.5 [83–90] years). Genotyping, determination of the minimum equilibrium concentration of rivaroxaban ($C_{min, ss}$), with standardization for the daily dose ($C_{min, ss/D}$), coagulogram and analysis of medical documentation for the presence of clinically significant non-major bleeding (CSNMB) were carried out. DDI was analyzed according to ABCB1 genotype.*
Results. *The use of rivaroxaban with verapamil in comparison with patients not taking calcium channel blockers (CCBs) leads to high $C_{min, ss}$ values in the CC genotype (rs1045642, rs4148738); $C_{min, ss}$ and $C_{min, ss}$/D in the CT genotype (rs1045642); prothrombin time in the CC genotype (rs1045642), more frequent occurrence of CRNM in the TT genotype (rs1045642, rs4148738). In comparison with patients taking amlodipine, it leads to high $C_{min, ss}$ values in the CT genotype (rs1045642), a more frequent occurrence of CSNMB in the TT genotype (rs1045642, rs4148738). The use of rivaroxaban with amlodipine in comparison with patients not taking CCBs leads to high $C_{min, ss}$ and $C_{min, ss}$/D values in the CC genotype (rs1045642) ($p < 0.017$).*
Conclusion. *The use of verapamil with rivaroxaban in ABCB1 TT carriers (rs4148738 and rs4148738) leads to the development of CSNMB in 75 and 78 % of cases, respectively. In patients taking rivaroxaban, it is advisable to test the ABCB1 genotype (rs4148738 and rs4148738) before adding a P-gp inhibitor to therapy.*

Key words: *drug-drug interactions, rivaroxaban, verapamil, ABCB1 (rs1045642 and rs4148738), therapeutic drug monitoring, older patients*

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ОСОБЕННОСТИ МЕЖЛЕКАРСТВЕННОГО ВЗАИМОДЕЙСТВИЯ РИВАРОКСАБАНА И БЛОКАТОРОВ КАЛЬЦИЕВЫХ КАНАЛОВ В ЗАВИСИМОСТИ ОТ ГЕНОТИПА *ABCB1* (rs1045642 И rs4148738) У ПАЦИЕНТОВ 80 ЛЕТ И СТАРШЕ С НЕКЛАПАННОЙ ФИБРИЛЛЯЦИЕЙ ПРЕДСЕРДИЙ

Сычев Д.А. [1],
Мирзаев К.Б. [1],
Черняева М.С. [2, 3],
Шахгильдян Н.В. [4],
Абдуллаев Ш.П. [1],
Денисенко Н.П. [1],
Созаева Ж.А. [1],
Качанова А.А. [1],
Шастина В.Р. [3, 5],
Горбатенкова С.В. [3]

[1] ФГБОУ ДПО «Российская медицинская академия непрерывного профессионального образования» Минздрава России (125993, г. Москва, ул. Баррикадная, 2/1, стр. 1, Россия)
[2] ФГБУ ДПО «Центральная государственная медицинская академия» Управления делами Президента Российской Федерации (121359, г. Москва, ул. Маршала Тимошенко, 19, стр. 1А, Россия)
[3] ГБУЗ «Госпиталь для ветеранов войн № 2 Департамента здравоохранения города Москвы» (109472, г. Москва, Волгоградский просп., 168, Россия)
[4] ФГБОУ ВО «Московский государственный университет имени М.В. Ломоносова» (119991, г. Москва, Ленинские горы, 1, Россия)
[5] ФГАОУ ВО «Первый Московский государственный медицинский университет имени И.М. Сеченова» Минздрава России (Сеченовский Университет) (119991, г. Москва, ул. Трубецкая, 8, стр. 2, Россия)

Автор, ответственный за переписку:
Черняева Марина Сергеевна,
e-mail: doctor@cherniaeva.ru

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РЕЗЮМЕ

Обоснование. *Применение ингибиторов гликопротеина P (P-gp) и носительство определённых полиморфизмов ABCB1 могут привести к увеличению концентрации ривароксабана и развитию кровотечений.*
Цель работы. *Изучить особенности межлекарственного взаимодействия (МЛВД) ривароксабана у пациентов старше 80 лет с неклапанной фибрилляцией предсердий в зависимости от генотипа ABCB1 (rs1045642 и rs4148738) на примере верапамила (ингибитор P-gp) и амлодипина.*
Материалы и методы. *Обследовано 128 пациентов (медиана возраста – 87,5 [83–90] лет). Проведены генотипирование, определение минимальной равновесной концентрации ривароксабана ($C_{min, ss}$) со стандартизацией на суточную дозу ($C_{min, ss}/D$), коагулограмма и анализ медицинской документации на наличие клинически значимых небольших кровотечений (КЗНК). Анализ по МЛВД проводился в зависимости от генотипа ABCB1.*
Результаты*. Применение ривароксабана с верапамилом в сравнении с пациентами, не принимающими блокаторы кальциевых каналов (БКК), приводит к высоким значениям $C_{min, ss}$ у генотипа CC (rs1045642, rs4148738); $C_{min, ss}$ и $C_{min, ss}/D$ – у генотипа CT (rs1045642); протромбинового времени – у генотипа CC (rs1045642); более частому возникновению КЗНК у генотипа TT (rs1045642, rs4148738). В сравнении с пациентами, принимающими амлодипин, применение ривароксабана с верапамилом приводит к высоким значениям $C_{min, ss}$ у генотипа CT (rs1045642), более частому возникновению КЗНК у генотипа TT (rs1045642, rs4148738). Применение ривароксабана с амлодипином в сравнении с пациентами, не принимающими БКК, приводит к высоким значениям $C_{min, ss}$ и $C_{min, ss}/D$ у генотипа CC (rs1045642) ($p < 0,017$).*
Заключение*. Применение верапамила с ривароксабаном у носителей генотипа TT ABCB1 (rs4148738 и rs4148738) приводит к развитию КЗНК в 75 % и 78 % случаев соответственно. У пациентов, принимающих ривароксабан, целесообразно исследование генотипа ABCB1 (rs4148738 и rs4148738) перед добавлением к терапии ингибитора P-gp.*

Ключевые слова: *межлекарственные взаимодействия, ривароксабан, верапамил, ABCB1 (rs1045642 и rs4148738), терапевтический лекарственный мониторинг, пожилые пациенты*

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RELEVANCE

Rivaroxaban is a substrate of the glycoprotein P (P-gp) transfer protein encoded by the *ABCB1* gene, which regulates the absorption of rivaroxaban from the lumen of the gastrointestinal tract and is also involved in its excretion through the liver and kidneys. Additionally, rivaroxaban is metabolised by cytochrome P-450 (CYP450), mainly by CYP3A4, with a minor contribution from CYP2J2 [1, 2]. Co-administration of medicinal products (MPs) that inhibit these metabolic pathways may increase rivaroxaban concentrations and increase the risk of adverse reactions (ARs), including bleeding [3]. In our previous study, we observed that co-administration of verapamil (a strong P-gp inhibitor and moderate CYP3A4 inhibitor) in combination with rivaroxaban resulted in a higher minimum equilibrium concentration ($C_{min, ss}$) of rivaroxaban compared with controls (Me, 73.8 [50.6–108.8] vs. 40.5 [25.6–74.3] ng/ml, respectively; $p = 0.003$) and, as a consequence, to more frequent AR in the form of small clinically significant non-major bleeding (CSNMB) (33 % vs. 13 %, respectively; $p = 0.036$) [4].

Genetic factors can also influence P-gp activity. Numerous SNP (single nucleotide polymorphism) have been observed in the *ABCB1* gene encoding P-gp, which may be associated with variations in P-gp expression and activity in humans during *in vitro* cell line studies [5–8]. *ABCB1* polymorphisms and haplotypes have been associated with changes in MP distribution and the development of AR to various MP substrates [9–11], although opinions are still conflicting [12, 13]. Although earlier in our study we did not obtain statistically significant differences in the pharmacokinetic profile in patients 80 years and older with non-valvular atrial fibrillation (AF) caused-specific to *ABCB1* gene polymorphisms (rs1045642 and rs4148738), nevertheless, we showed that AR in the form of clinically significant small hemorrhages was more frequent in patients carrying the homozygous type (TT) of the *ABCB1* gene (rs1045642) compared to patients carrying the wild type (CC) (29.3 % vs. 4.5 % of cases; $p < 0.050$) and prothrombin time (PT) was statistically significantly higher. Patients carrying the homozygous type (TT) of the *ABCB1* (rs4148738) gene were statistically significantly more likely to have clinically significant minor bleeding compared to patients carrying the wild type (CC) (39.3 % vs. 8.1 % of cases; $p < 0.050$) and heterozygous type (CT) (39.3 % vs. 14.3 % of cases; $p < 0.050$) of the *ABCB1* (rs4148738) gene [14].

Therefore, as a continuation of our studies, it was decided to separately analyse the pharmacokinetic profile of patients carrying each of the genotypes (CC, CT and TT) of the *ABCB1* gene (rs1045642 and rs4148738) in relation to concomitant therapy with calcium channel blockers (CCBs), where co-administration of rivaroxaban (P-gp substrate) with amlodipine (dihydropyridine BCA (DCCB)) (P-gp substrate) may lead to drug interaction caused by competition between the substrates for binding sites on cell membranes, and co-administration of rivaroxaban with verapamil (non-DCCB) (strong P-gp inhibitor and moderate CYP3A4 inhibitor) may lead to drug interactions as a result by inhibition of the leading transport and metabolic pathways of rivaroxaban.

THE AIM OF THE STUDY

To study the features of drug interaction between rivaroxaban in patients over 80 years old with non-valvular atrial fibrillation caused-specific to *ABCB1* genotype (rs1045642 and rs4148738) using verapamil (P-gp inhibitor) and amlodipine as an example.

MATERIALS AND METHODS

Study design and ethics

A cross-sectional study of patients 80 years and older with non-valvular AF recruited between January 2019 and February 2020 was conducted. The study was approved by the Ethical Committee of the Russian Medical Academy of Continuous Professional Education (Protocol No. 1 dated January 22, 2019) and was conducted in accordance with the World Medical Association's Declaration of Helsinki in compliance with the rules of good clinical practice. Verbal and written informed consent was obtained from all participants included in the study.

Patients

We examined 128 patients older than 80 years (median age – 87.5 [83–90] years; 75 % women) of Caucasian race with non-valvular AF who were under treatment in a multidisciplinary hospital in Moscow. Patients were consecutively included in the study if they met the inclusion criteria. Inclusion criteria: 1) patients with non-valvular AF of both sexes; 2) age at the time of inclusion in the study – 80 years and older; 3) duration of previous intake of rivaroxaban with verapamil, amlodipine or without CCB – at least 1 year from the time of inclusion in the study; 4) signing a voluntary informed consent for participation in the study. The main exclusion criteria were 1) age less than 80 years; 2) concomitant drug therapy with known drug interactions with rivaroxaban (fluconazole, ketoconazole and other azole antifungal drugs; ritonavir and other human immunodeficiency virus protease inhibitors; amiodarone, clarithromycin, erythromycin; platelet aggregation inhibitors (including acetylsalicylic acid); non-steroidal anti-inflammatory drugs; selective serotonin and norepinephrine reuptake inhibitors; rifampicin, phenytoin, carbamazepine, phenobarbital, *Hypericum perforatum* preparations); 3) patient's violation of the procedures of the examination and treatment plan; 4) refusal to participate in the study.

All patients were taking rivaroxaban (once-daily) for ischemic stroke prophylaxis at a dose of 15 mg/day (86.7 % of patients) and 20 mg/day (13.3 % of patients). Each patient was genotyped for the examined polymorphism and the minimum equilibrium concentration of ri-

varoxaban ($C_{min, ss}$) was determined. An additional standardisation of the minimum equilibrium concentration of rivaroxaban per daily drug dose ($C_{min,ss}$/D) was performed. Furthermore, all patients underwent clinical and biochemical blood tests, urinalysis, coagulogram with determination of PT in plasma. Medical records were analysed for the presence of AR in the form of CSNMB bleeding against the background of rivaroxaban intake during the previous year from the time of inclusion in the study [15, 16].

Genotyping

The material for DNA extraction was venous blood, which was sampled in 4 ml Vacuette® vacuum tubes with K3 EDTA anticoagulant. Genotyping by polymorphisms rs1045642 and rs4148738 of the *ABCB1* gene was performed using real-time polymerase chain reaction on a CFX96 Touch™ Real-Time PCR Detection System DNA amplifier (Bio-Rad Laboratories Inc., USA) at the Research Institute of Molecular and Personalised Medicine, Russian Medical Academy of Continuing Professional Education, Ministry of Health of Russia.

Determination of rivaroxaban concentration in plasma

Venous blood was sampled to determine $C_{min, ss}$ of rivaroxaban on day 7 of administering a fixed dose of anticoagulant (at least 5 half-lives) immediately before the next dose of medicinal product. Determination of $C_{min, ss}$ of rivaroxaban in blood was performed by high-performance liquid chromatography with mass spectrometric detection. Samples were analysed on an Agilent 1200 liquid chromatograph (Agilent Technologies, USA) (four-channel pump, mobile phase degasser, chromatographic column thermostat). Agilent Extend-C18 column (Agilent Technologies, USA) (length 100 mm, inner diameter 2.1 mm, grain size 3.5 µm) was used in this study. The methodology is described in more detail in our previous study [14].

Laboratory tests

Venous blood for coagulogram determination, clinical blood count and urinalysis (morning portion) was collected at the same time as blood collection for measurement of $C_{min, ss}$ of rivaroxaban. Coagulogram was determined using an automatic coagulometer ACL Elite Pro (Instrumentation Laboratory, USA); clinical blood analysis was performed on a haematological analyser ADVIA® 2120i (Siemens, USA), biochemical blood analysis was performed using an integrated analyser for biochemical, immunochemical and electrolyte analysis Siemens Dimension X and Plus (Siemens, USA); total urine analysis was performed on an automated urine analyser Aution Max™ AX-4280 Automated Urine Chemistry Analyzer (ARKRAY Factory Inc., Russia). All studies were performed according to the manufacturer's instructions. The glomerular filtration rate was calculated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula [17].

Statistical data processing was performed in the IBM SPSS Statistics 26 software package (IMB Corp., USA). Sample description for non-normally distributed indicators was performed by calculating the median (Me) and interquartile range as the 25th and 75th percentiles (C25 and C75); for normally distributed indicators – by determining the mean (Mean) with standard deviation (SD, standard deviation). The normality of the distribution of the obtained parameters was evaluated using the Shapiro – Wilk criterion. For non-normally distributed indicators, the non-parametric Mann – Whitney U test was applied; categorical data were evaluated using Fisher's exact test, where differences are considered statistically significant at $p < 0.050$. To reduce the probability of occurrence of the first type errors in multiple comparisons, the statistical significance of differences was assessed with the Bonferroni correction, dividing the value of 0.05 by the number of comparisons (3) [18], with $p < 0.017$ considered to be the threshold value of statistical significance of differences.

RESULTS

Peculiarities of drug interaction between rivaroxaban and calcium channel blockers cause-specific to *ABCB1* (rs1045642) genotype

Cause-specific to the *ABCB1* genotype (rs1045642), patients were divided into three groups: group 1 – carriers of the wild-type CC genotype ($n = 22$); group 2 – carriers of the heterozygous CT genotype ($n = 65$); group 3 – carriers of the homozygous TT genotype ($n = 41$). Drug interactions were analysed within each group cause-specific to concomitant therapy: patients taking rivaroxaban without CCB were assumed to have no drug interactions; patients taking rivaroxaban + amlodipine DCCB had a potentially possible drug interaction caused by substrate competition for binding sites on cell membranes; in patients taking rivaroxaban + non-DCCB (verapamil), a strong P-gp inhibitor, these drugs may lead to drug interactions as a result of inhibition of the leading transport and metabolic pathways of rivaroxaban.

Group 1. Wild type (CC) carriers of the *ABCB1* gene (rs1045642)

We divided group 1 patients ($n = 22$) into subgroups cause-specific to concomitant therapy. Subgroup 1 – 7 patients taking rivaroxaban without CCB (median age – 91 [81.0–91.0] years; 71.4 % women); subgroup 2 – 11 patients taking rivaroxaban + amlodipine (median age – 89.0 [81.0–90.0] years; 90.9 % women); subgroup 3 – 4 patients taking rivaroxaban + verapamil (median age – 90.5 [85.3–93.5] years; 100 % women). The baseline characteristics of the patients included in the subgroups are presented in Table 1. Patients in subgroup 3 (rivaroxaban + verapamil) were more likely to have a history of stroke than patients in subgroup 1 (rivaroxaban without CCB). In other parameters, patients in the subgroups studied were comparable.

In patients carrying the wild-type (WS) *ABCB1* gene (rs1045642), the $C_{min, ss}$ levels of rivaroxaban were statistically significantly higher in subgroup 3 (rivaroxaban + verapamil) compared with subgroup 1 (rivaroxaban without CCB) ($p = 0.014$) and statistically significantly higher in subgroup 2 (rivaroxaban + amlodipine) compared with subgroup 1 ($p = 0.002$).

In subgroup 2 (rivaroxaban + amlodipine), the $C_{min, ss}$/D levels of rivaroxaban were statistically significantly higher than in subgroup 1 (rivaroxaban without CCB) ($p = 0.023$).

PT levels in subgroup 3 (rivaroxaban + verapamil) were higher than in subgroup 1, (rivaroxaban without CCB) and subgroup 2 (rivaroxaban + amlodipine), but without reaching statistical significance of differences $p = 0.059$ and $p = 0.358$, respectively) (Table 2).

Group 2. Heterozygous type (CT) carriers of the *ABCB1* gene (rs1045642)

We divided group 2 patients ($n = 65$) into 3 subgroups cause-specific to concomitant therapy. Subgroup 1 – 28 patients taking rivaroxaban without CCB (median age – 88.0 [84.0–90.0] years; 71.4 % women); subgroup 2 – 23 patients taking rivaroxaban + amlodipine (median age – 85.0 [83.0–88.0] years; 82.6 % of women); subgroup 3 – 14 patients taking rivaroxaban + verapamil (median age – 88.0 [81.0–89.3] years; 64.3 % of women). The baseline characteristics of the patients included in the subgroups are presented in Table 3. The patients in the study groups were comparable with respect to the main parameters.

Baseline characteristics of patients carrying the CT genotype of the *ABCB1* gene (rs1045642)

In patients carrying the heterozygous type (CT) of the *ABCB1* gene (rs1045642), the $C_{min, ss}$ levels of rivaroxaban were statistically significantly higher in subgroup 3 (rivaroxaban + verapamil) compared to subgroup 1 (rivaroxaban without CCB) ($p = 0.011$) and compared to subgroup 2 (rivaroxaban + amlodipine) ($p = 0.017$). Rivaroxaban $C_{min, ss}$/D levels were statistically significantly higher in subgroup 3 (rivaroxaban + verapamil) compared to subgroup 1 (rivaroxaban without CCB) ($p = 0.014$) (Table 4).

No differences in PT and the number of patients who experienced AR in the form of clinically significant minor

TABLE 1

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE CC GENOTYPE OF *ABCB1* GENE (rs1045642)

Indicators	CC genotype ($n = 22$) of the *ABCB1* gene (rs1045642)					
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	*p* value
Number of patients, abs. (%)	7/22 (31.8)	11/22 (50)	4/22 (18.2)	18/22 (81.8)	15/22 (68.2)	–
Age (years), Me [C25–C75]	91.0 [81.0–91.0]	89.0 [81.0–90.0]	90.5 [85.3–93.5]	89.5 [81.0–91.0]	89.0 [84.0–91.0]	$p_4 = 0.596$ $p_5 = 0.315$ $p_6 = 0.280$ $p_7 = 0.262$ $p_8 = 1.000$
Women, abs. (%)	5/7 (71.4)	10/11 (90.9)	4/4 (100)	18/18 (100)	14/15 (93.3)	$p_4 = 0.280$ $p_5 = 0.237$ $p_6 = 0.533$ $p_7 = 0.380$ $p_8 = 0.163$
BMI (kg/m^2), Me [C25–C75]	27.2 [24.1–30.3]	25.6 [24.2–29.8]	29.3 [22.1–33.1]	25.6 [24.3–29.5]	26.5 [24.2–31.4]	$p_4 = 0.792$ $p_5 = 0.762$ $p_6 = 0.539$ $p_7 = 0.554$ $p_8 = 1.000$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	5.0 [3.0–6.0]	6.0 [5.3–6.0]	7.0 [4.5–8.8]	6.0 [5.0–6.0]	6.0 [5.3–6.0]	$p_4 = 0.282$ $p_5 = 0.257$ $p_6 = 0.461$ $p_7 = 0.277$ $p_8 = 0.180$
HAS-BLED (scores), Me [C25–C75]	4.5 [3.0–6.0]	3.0 [3.0–3.8]	4.5 [3.0–5.0]	3.0 [3.0–6.0]	3.0 [3.0–4.0]	$p_4 = 0.081$ $p_5 = 0.762$ $p_6 = 0.073$ $p_7 = 0.382$ $p_8 = 0.151$

TABLE 1 (*continued*)

Number of MPs (scores), Me [C25–C75]	6.0 [5.0–8.0]	6.0 [5.0–9.0]	6.0 [5.3–7.5]	6.0 [5.0–8.3]	6.0 [5.0–8.0]	$p_4 = 1.000$ $p_5 = 1.000$ $p_6 = 0.851$ $p_7 = 0.837$ $p_8 = 1.000$
Number of MPs ≥ 5, abs. (%)	6/7 (85.7)	11/11 (100)	4/4 (100)	17/18 (94.4)	15/15 (100)	$p_4 = 0.197$ $p_5 = 0.428$ $p_6 = –$ $p_7 = 0.629$ $p_8 = 0.134$
Dose of rivaroxaban 15 mg, abs. (%)	6/7 (85.7)	9/11 (81.8)	3/4 (75)	15/18 (83.3)	12/15 (80)	$p_4 = 0.829$ $p_5 = 0.658$ $p_6 = 0.770$ $p_7 = 0.696$ $p_8 = 0.746$
Dose of rivaroxaban 20 mg, abs. (%)	1/7 (14.3)	2/11 (18.2)	1/4 (25)	3/18 (16.7)	3/15 (20)	$p_4 = 0.829$ $p_5 = 0.658$ $p_6 = 0.770$ $p_7 = 0.696$ $p_8 = 0.746$
Creatinine (μmol/L), Me [C25–C75]	96.8 [79.4–104.0]	97.4 [82.8–129.5]	91.8 [58.9–106.9]	97.3 [82.3–109.0]	96.4 [82.8–111.8]	$p_4 = 0.497$ $p_5 = 0.648$ $p_6 = 0.343$ $p_7 = 0.386$ $p_8 = 0.856$
Hemoglobin (g/L), Me [C25–C75]	116.0 [112.0–147.0]	118.0 [112.0–126.0]	120.5 [108.0–132.3]	117.0 [112.0–131.3]	118.0 [112.0–127.0]	$p_4 = 0.536$ $p_5 = 0.648$ $p_6 = 0.753$ $p_7 = 1.000$ $p_8 = 0.490$
Platelets (10^9/L), Me [C25–C75]	248.0 [191.0–263.0]	245.0 [192.0–305.0]	164.5 [156.0–254.8]	246.5 [191.8–271.5]	245.0 [188.0–283.0]	$p_4 = 0.536$ $p_5 = 0.527$ $p_6 = 0.078$ $p_7 = 0.141$ $p_8 = 0.837$
Comorbidities						
CHD, abs. (%)	7/7 (100)	11/11 (100)	4/4 (100)	18/18 (100)	15/15 (100)	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
Effort-induced angina pectoris, abs. (%)	6/7 (85.7)	10/11 (90.9)	4/4 (100)	16/18 (88.9)	14/15 (93.3)	$p_4 = 0.732$ $p_5 = 0.428$ $p_6 = 0.360$ $p_7 = 0.533$ $p_8 = 1.000$
Heart failure, abs. (%)	7/7 (100)	11/11 (100)	4/4 (100)	18/18 (100)	15/15 (100)	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
Arterial hypertension, abs. (%)	6/7 (85.7)	9/11 (81.8)	4/4 (100)	15/18 (83.3)	13/15 (86.7)	$p_4 = 0.829$ $p_5 = 0.428$ $p_6 = 0.360$ $p_7 = 0.380$ $p_8 = 0.952$
History of previous myocardial infarction, abs. (%)	2/7 (28.6)	9/11 (81.8)	2/4 (50)	8/18 (44.4)	8/15 (53.3)	$p_4 = 0.280$ $p_5 = 0.477$ $p_6 = 0.876$ $p_7 = 0.840$ $p_8 = 0.277$

TABLE 1 (*continued*)

ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	0/7 (0)	2/11 (18.2)	3/4 (75)	2/18 (11.1)	5/15 (33.3)	$p_4 = 0.231$ $p_5 = 0.007$ $p_6 = 0.039$ $p_7 = 0.006$ $p_8 = 0.082$
Bronchial asthma, abs. (%)	0/7 (0)	0/11 (0)	1/4 (25)	0/18 (0)	1/15 (6.7)	$p_4 = –$ $p_5 = 0.165$ $p_6 = 0.086$ $p_7 = 0.030$ $p_8 = 0.484$
Lower extremity atherosclerosis, abs. (%)	0/7 (0)	0/11 (0)	1/4 (25)	0/18 (0)	1/15 (6.7)	$p_4 = –$ $p_5 = 0.165$ $p_6 = 0.086$ $p_7 = 0.030$ $p_8 = 0.484$
Chronic bronchitis, abs. (%)	3/7 (42.9)	4/11 (36.4)	0/4 (0)	7/18 (38.9)	4/15 (26.7)	$p_4 = 0.783$ $p_5 = 0.125$ $p_6 = 0.159$ $p_7 = 0.131$ $p_8 = 0.448$
Type 2 diabetes mellitus, abs. (%)	3/7 (42.9)	3/11 (27.3)	0/4 (0)	6/18 (33.3)	3/15 (20)	$p_4 = 0.494$ $p_5 = 0.125$ $p_6 = 0.243$ $p_7 = 0.176$ $p_8 = 0.262$
Charlson's comorbidity index (abs.), Me [C25–C75]	11.0 [9.0–12.0]	10.0 [9.0–12.0]	11.0 [10.3–14.0]	10 [9–12]	10.0 [9.0–12.0]	$p_4 = 0.860$ $p_5 = 0.527$ $p_6 = 0.280$ $p_7 = 0.300$ $p_8 = 0.945$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25–C75]	45.8 [41.9–59.9]	44.1 [32.9–55.3]	47.2 [38.2–76.6]	44.1 [41.2–56.1]	44.8 [36.7–55.3]	$p_4 = 0.246$ $p_5 = 1.000$ $p_6 = 0.412$ $p_7 = 0.594$ $p_8 = 0.488$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	0/7 (0)	2/11 (18.2)	0	2/18 (11.1)	2/15 (13.3)	$p_4 = 0.231$ $p_5 = –$ $p_6 = 0.360$ $p_7 = 0.484$ $p_8 = 0.293$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	–	20.5 [19.5–20.5]	–	20.5 [19.5–20.5]	20.4 [19.5–20.4]	–
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	3/7 (42.9)	5/11 (45.5)	1/4 (25)	8/18 (44.4)	5/15 (33.3)	$p_4 = 0.914$ $p_5 = 0.554$ $p_6 = 0.475$ $p_7 = 0.474$ $p_8 = 0.751$
GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	41.9 [40.4–41.9]	42.8 [37.9–44.1]	36.59	42.3 [39.4–44.1]	42.7 [36.6–44.1]	$p_4 = 1.000$ $p_5 = 0.500$ $p_6 = 0.333$ $p_7 = 0.222$ $p_8 = 1.000$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	2/7 (28.6)	3/11 (27.3)	2/4 (50)	5/18 (27.8)	5/15 (33.3)	$p_4 = 0.952$ $p_5 = 0.477$ $p_6 = 0.409$ $p_7 = 0.388$ $p_8 = 0.751$

TABLE 1 (*continued*)

GFR in CKD Stage 3A (ml/min/1.73 m^2), Me [C25–C75]	50.6 [45.8–50.6]	54.8 [45.5–54.8]	47.2 [45.9–47.2]	54.8 [45.6–56.1]	48.5 [45.7–55.8]	$p_4 = 1.000$ $p_5 = 1.000$ $p_6 = 1.000$ $p_7 = 0.857$ $p_8 = 0.293$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	1/7 (14.3)	1/11 (9.1)	1/4 (25)	2/18 (11.1)	2/15 (13.3)	$p_4 = 0.732$ $p_5 = 0.658$ $p_6 = 0.432$ $p_7 = 0.464$ $p_8 = 0.571$
GFR in CKD Stage 2 (ml/min/1.73 m^2), Me [C25–C75]	59.87	81.1	85.97	70.5 [59.9–70.5]	83.5 [81.1–83.5]	$p_4 = 1.000$ $p_5 = 1.000$ $p_6 = 1.000$ $p_7 = 1.000$ $p_8 = 1.000$
CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	1/7 (14.3)	–	–	1/18 (5.6)	0/15 (0)	$p_4 = 0.197$ $p_5 = 0.428$ $p_6 = –$ $p_7 = 0.629$ $p_8 = 0.147$
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	96.96	–	–	96.96	–	–

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

TABLE 2

PECULIARITIES OF DRUG INTERACTION IN PATIENTS CARRYING THE CC GENOTYPE OF THE *ABCB1* GENE (rs1045642)

Indicators	CC genotype ($n = 22$) of the *ABCB1* gene (rs1045642)			*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	7/22 (31.8)	11/22 (50)	4/22 (18.2)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	15.3 [12.0–28.4]	69.4 [45.6–100.8]	67.8 [45.0–76.9]	$p_4 = 0.002$ $p_5 = 0.014$ $p_6 = 0.602$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	1.0 [0.8–1.9]	4.6 [3.0–6.4]	4.0 [2.8–5.1]	$p_4 = 0.003$ $p_5 = 0.023$ $p_6 = 0.647$
Prothrombin time (s), Me [C25–C75]	12.6 [12.3–13.8]	13.4 [12.3–14.5]	14.2 [12.9–18.3]	$p_4 = 0.388$ $p_5 = 0.059$ $p_6 = 0.358$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	1/7 (14.3)	0/11 (0)	0/4 (0)	$p_4 = 0.197$ $p_5 = 0.428$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

bleeding were observed between the compared subgroups ($p > 0.017$) (Table 4).

Group 3. Homozygous type (TT) carriers of the *ABCB1* gene (rs1045642)

We divided patients in group 3 ($n = 41$) into 3 subgroups cause-specific to concomitant therapy. Subgroup 1 – 12 patients taking rivaroxaban without CCB (median age – 88.0 [83.0–88.8] years; 75 % women); subgroup 2 – 17 patients taking rivaroxaban + amlodipine (median age – 86.0 [82.0–88.0] years; 88.2 % women); subgroup 3 – 12 patients taking rivaroxaban + verapamil (median age – 89.5 [85.5–92.0] years; 41.7 % women). The baseline characteristics of the patients inclu-

TABLE 3

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE CT GENOTYPE OF THE *ABCB1* GENE (rs1045642)

Indicators	CT genotype ($n = 65$) of the *ABCB1* gene (rs1045642)					
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	*p* value
Number of patients, abs. (%)	28/65 (43.1)	23/65 (35.4)	14/65 (21.5)	51/65 (78.5)	37/65 (56.9)	–
Age (years), Me [C25–C75]	88.0 [84.0–90.0]	85.0 [83.0–88.0]	88.0 [81.0–89.3]	87 [83–90]	86.0 [82.0–89.0]	$p_4 = 0.243$ $p_5 = 0.308$ $p_6 = 0.865$ $p_7 = 0.481$ $p_8 = 0.184$
Women, abs. (%)	20/28 (71.4)	19/23 (82.6)	9/14 (64.3)	39/51 (76.5)	28/37 (75.7)	$p_4 = 0.349$ $p_5 = 0.637$ $p_6 = 0.208$ $p_7 = 0.358$ $p_8 = 0.700$
BMI (kg/m^2), Me [C25–C75]	28.5 [26.0–31.1]	30.5 [26.9–32.3]	30.0 [25.4–32.0]	30.1 [26.2–31.2]	30.5 [26.6–32.1]	$p_4 = 0.271$ $p_5 = 0.424$ $p_6 = 0.624$ $p_7 = 0.805$ $p_8 = 0.237$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	6.0 [5.0–7.0]	6.0 [5.0–7.3]	7.0 [5.0–8.0]	6 [5–7]	8.0 [5.0–8.0]	$p_4 = 0.321$ $p_5 = 0.145$ $p_6 = 0.531$ $p_7 = 0.244$ $p_8 = 0.157$
HAS-BLED (scores), Me [C25–C75]	3.0 [2.0–4.0]	3.0 [3.0–3.5]	3.0 [2.0–4.0]	3 [2–4]	3.0 [2.5–4.0]	$p_4 = 0.812$ $p_5 = 0.945$ $p_6 = 0.932$ $p_7 = 0.923$ $p_8 = 0.866$
Number of MPs (scores), Me [C25–C75]	6.0 [5.0–7.0]	8.0 [6.0–9.0]	6.0 [5.8–7.0]	6 [5–8]	7.0 [6.0–8.0]	$p_4 = 0.03$ $p_5 = 0.452$ $p_6 = 0.028$ $p_7 = 0.506$ $p_8 = 0.012$
Number of MPs ≥ 5, abs. (%)	23/28 (82.1)	22/23 (95.7)	12/14 (85.7)	45/51 (88.2)	34/37 (91.9)	$p_4 = 0.136$ $p_5 = 0.770$ $p_6 = 0.283$ $p_7 = 0.799$ $p_8 = 0.124$
Dose of rivaroxaban 15 mg, abs. (%)	25/28 (89.3)	19/23 (82.6)	13/14 (92.9)	44/51 (86.3)	32/37 (86.5)	$p_4 = 0.491$ $p_5 = 0.710$ $p_6 = 0.377$ $p_7 = 0.507$ $p_8 = 0.734$
Dose of rivaroxaban 20 mg, abs. (%)	3/28 (10.7)	4/23 (17.4)	1/14 (7.1)	7/51 (13.7)	4/37 (10.8)	$p_4 = 0.491$ $p_5 = 0.710$ $p_6 = 0.377$ $p_7 = 0.507$ $p_8 = 0.990$

TABLE 3 (*continued*)

Creatinine (μmol/L), Me [C25–C75]	108.0 [92.9–130.8]	94.8 [86.0–110.0]	99.6 [83.4–117.5]	101 [90.3–123]	97.0 [85.8–113.5]	$p_4 = 0.065$ $p_5 = 0.249$ $p_6 = 0.963$ $p_7 = 0.503$ $p_8 = 0.061$
Hemoglobin (g/L), Me [C25–C75]	123.0 [112.5–135.3]	125.0 [11.0–132.0]	127.0 [116.5–134.3]	125 [112–133]	125.0 [112.0–133.0]	$p_4 = 0.726$ $p_5 = 0.823$ $p_6 = 0.506$ $p_7 = 0.626$ $p_8 = 0.895$
Platelets (10^9/L), Me [C25–C75]	222.0 [165.3–260.3]	217.0 [190.0–293.0]	203.0 [169.5–257.5]	219 [175–264]	215.0 [176.0–274.0]	$p_4 = 0.449$ $p_5 = 0.947$ $p_6 = 0.546$ $p_7 = 0.714$ $p_8 = 0.624$
Comorbidities						
CHD, abs. (%)	28/28 (100)	23/23 (100)	14/14 (100)	51/51 (100)	37/37 (100)	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
Effort-induced angina pectoris, abs. (%)	24/28 (85.7)	20/23 (87.0)	12/14 (85.7)	44/51 (86.3)	32/37 (86.5)	$p_4 = 0.898$ $p_5 = 1.000$ $p_6 = 0.915$ $p_7 = 0.957$ $p_8 = 0.929$
Heart failure, abs. (%)	27/28 (96.4)	21/23 (91.3)	12/14 (85.7)	48/51 (94.1)	33/37 (89.2)	$p_4 = 0.439$ $p_5 = 0.204$ $p_6 = 0.595$ $p_7 = 0.296$ $p_8 = 0.278$
Arterial hypertension, abs. (%)	27/28 (96.4)	22/23 (95.7)	13/14 (92.9)	49/51 (96.1)	35/37 (94.6)	$p_4 = 0.887$ $p_5 = 0.608$ $p_6 = 0.715$ $p_7 = 0.611$ $p_8 = 0.727$
History of previous myocardial infarction, abs. (%)	5/28 (17.9)	4/23 (17.4)	2/14 (14.3)	9/51 (17.6)	6/37 (16.2)	$p_4 = 0.965$ $p_5 = 0.770$ $p_6 = 0.804$ $p_7 = 0.766$ $p_8 = 0.861$
ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	6/28 (21.4)	5/23 (21.7)	3/14 (21.4)	11/51 (21.6)	8/37 (21.6)	$p_4 = 0.979$ $p_5 = 1.000$ $p_6 = 0.982$ $p_7 = 0.991$ $p_8 = 0.985$
Bronchial asthma, abs. (%)	0/28 (0)	1/23 (4.3)	2/14 (14.3)	1/51 (2)	3/37 (8.1)	$p_4 = 0.265$ $p_5 = 0.040$ $p_6 = 0.283$ $p_7 = 0.052$ $p_8 = 0.123$
Lower extremity atherosclerosis, abs. (%)	3/28 (10.7)	5/23 (21.7)	3/14 (21.4)	8/51 (15.7)	8/37 (21.6)	$p_4 = 0.281$ $p_5 = 0.350$ $p_6 = 0.982$ $p_7 = 0.612$ $p_8 = 0.246$
Chronic bronchitis, abs. (%)	10/28 (35.7)	8/23 (34.8)	6/14 (42.9)	18/51 (35.3)	14/37 (37.8)	$p_4 = 0.045$ $p_5 = 0.653$ $p_6 = 0.623$ $p_7 = 0.603$ $p_8 = 0.861$
Type 2 diabetes mellitus, abs. (%)	8/28 (28.6)	7/23 (30.4)	4/14 (28.6)	15/51 (29.4)	11/37 (29.7)	$p_4 = 0.884$ $p_5 = 1.000$ $p_6 = 0.904$ $p_7 = 0.951$ $p_8 = 0.919$

TABLE 3 (*continued*)

Charlson's comorbidity index (abs.), Me [C25–C75]	10.0 [9.0–11.0]	10.0 [9.0–12.0]	10.0 [9.0–12.0]	10 [9–11]	10.0 [9.0–12.0]	$p_4 = 0.869$ $p_5 = 0.927$ $p_6 = 0.841$ $p_7 = 0.864$ $p_8 = 0.946$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25–C75]	42.8 [36.0–49.8]	46.5 [40.5–54.2]	51.7 [36.8–56.1]	48.0 [37.1–57.6]	47.8 [38.9–54.4]	$p_4 = 0.153$ $p_5 = 0.119$ $p_6 = 0.632$ $p_7 = 0.231$ $p_8 = 0.075$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	2/28 (7.1)	2/23 (8.7)	1/14 (7.1)	4/51 (7.8)	4/37 (10.8)	$p_4 = 0.837$ $p_5 = 1.000$ $p_6 = 0.867$ $p_7 = 0.931$ $p_8 = 0.990$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	24.3 [19.3–24.3]	27.0 [24.6–27.0]	26.4	26.9 [20.7–29.4]	27.8 [25.0–29.6]	$p_4 = 0.667$ $p_5 = 1.000$ $p_6 = 1.000$ $p_7 = 1.000$ $p_8 = 0.857$
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	13/28 (46.4)	7/23 (30.4)	4/14 (64.3)	20/51 (39.2)	10/37 (27.0)	$p_4 = 0.244$ $p_5 = 0.116$ $p_6 = 0.550$ $p_7 = 0.218$ $p_8 = 0.182$
GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	37.8 [32.5–39.1]	40.5 [33.2–41.9]	36.12 [34.3–42.8]	37.8 [32.9–41.6]	38.9 [34.6–42.1]	$p_4 = 0.485$ $p_5 = 0.521$ $p_6 = 0.517$ $p_7 = 0.457$ $p_8 = 0.497$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	12/28 (42.9)	11/23 (47.8)	9/14 (64.3)	23/51 (45.1)	19/37 (51.4)	$p_4 = 0.723$ $p_5 = 0.190$ $p_6 = 0.330$ $p_7 = 0.203$ $p_8 = 0.497$
GFR in CKD Stage 3A (ml/min/1.73 m^2), Me [C25–C75]	49.3 [46.4–53.1]	50.5 [46.6–54.2]	53.9 [47.9–56.4]	50.3 [46.6–53.7]	52.6 [47.8–54.9]	$p_4 = 0.525$ $p_5 = 0.247$ $p_6 = 0.412$ $p_7 = 0.246$ $p_8 = 0.177$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	1/28 (3.6)	3/23 (13)	1/14 (7.1)	4/51 (7.8)	4/37 (10.8)	$p_4 = 0.211$ $p_5 = 0.608$ $p_6 = 0.575$ $p_7 = 0.931$ $p_8 = 0.278$
GFR in CKD Stage 2 (ml/min/1.73 m^2), Me [C25–C75]	62.37	68.0 [63.3–68.0]	66.76	65.6 [62.6–71.7]	67.4 [64.1–71.7]	$p_4 = 0.500$ $p_5 = 1.000$ $p_6 = 1.000$ $p_7 = 1.000$ $p_8 = 0.400$
CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	0/28 (100)	0/23 (0)	0/14 (0)	0/51 (0)	0/37 (0)	–
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	–	–	–	–		–

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

ded in the subgroups are presented in Table 5. Patients in subgroup 3 (rivaroxaban + verapamil) had more men than patients in subgroup 2 (rivaroxaban + amlodipine); they were also more likely than patients in subgroup 1 (rivaroxaban without CCB) to have lower extremity atherosclerosis and had a higher Charlson comorbidity index. In other parameters, patients in the subgroups studied were comparable.

Patients carrying homozygous type (TT) of *ABCB1* gene (rs1045642) had baseline higher values of $C_{min, ss}$ of rivaroxaban, $C_{min, ss}$/D of rivaroxaban and PT, and no differences were found between the compared subgroups in these parameters ($p > 0.017$) (Table 6).

Along with this AR in the form of clinically significant minor bleeding occurred in 75 % (!) of cases in subgroup 3 (rivaroxaban + verapamil), and this rate was statistically significantly higher in comparison with subgroup 1 (rivaroxaban without CCB) ($p = 0.001$), and in comparison with subgroup 2 (rivaroxaban + amlodipine) ($p = 0.001$) (Table 6).

Peculiarities of drug interaction between rivaroxaban and calcium channel blockers cause-specific to *ABCB1* (rs4148738) genotype

Cause-specific to the *ABCB1* genotype (rs4148738), patients were divided into three groups: group 1 – carriers of the wild-type CC genotype ($n = 37$); group 2 – carriers of the heterozygous ST genotype ($n = 63$); group 3 – carriers of the homozygous TT genotype ($n = 28$). Drug interaction analyses were performed within each group cause-specific to concomitant therapy, in the same manner as for the previous polymorphism.

TABLE 4

PECULIARITIES OF DRUG INTERACTION IN PATIENTS CARRYING THE CT GENOTYPE OF THE *ABCB1* GENE (rs1045642)

Indicators	CT genotype ($n = 65$) of the *ABCB1* gene (rs1045642)			*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	28/65 (43.1)	23/65 (35.4)	14/65 (21.5)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	41.7 [25.7–70.7]	48.6 [28.2–65.5]	90.7 [51.7–140.8]	$p_4 = 0.910$ $p_5 = 0.011$ $p_6 = 0.017$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	2.7 [1.7–4.0]	3.2 [1.9–4.0]	6.1 [3.2–9.4]	$p_4 = 1.000$ $p_5 = 0.014$ $p_6 = 0.020$
Prothrombin time (s), Me [C25–C75]	14.0 [12.6–14.6]	13.2 [12.6–14.3]	14.6 [13.2–16.1]	$p_4 = 0.602$ $p_5 = 0.157$ $p_6 = 0.121$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	4/28 (14.3)	5/23 (21.7)	1/14 (7.1)	$p_4 = 0.487$ $p_5 = 0.500$ $p_6 = 0.243$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

TABLE 5

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE TT GENOTYPE OF *ABCB1* GENE (rs1045642)

Indicators	TT genotype ($n = 41$) of the *ABCB1* gene (rs1045642)					*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	
Number of patients, abs. (%)	12/41 (29.3)	17/41 (41.4)	12/41 (29.3)	29/41 (70.7)	29/41 (70.7)	–
Age (years), Me [C25–C75]	88.0 [83.0–88.8]	86.0 [82.0–88.0]	89.5 [85.5–92.0]	87 [82–88]	87.0 [83.0–90.0]	$p_4 = 0.394$ $p_5 = 0.319$ $p_6 = 0.08$ $p_7 = 0.106$ $p_8 = 0.944$

TABLE 5 (*continued*)

Women, abs. (%)	9/12 (75)	15/17 (88.2)	5/12 (41.7)	24/29 (82.8)	20/29 (69.0)	$p_4 = 0.353$ $p_5 = 0.098$ $p_6 = 0.008$ $p_7 = 0.009$ $p_8 = 0.699$
BMI (kg/m^2), Me [C25–C75]	26.6 [23.5–33.0]	27.5 [24.3–31.6]	25.6 [24.2–29.4]	27.4 [24.3–31.9]	26.9 [24.3–30.9]	$p_4 = 0.909$ $p_5 = 0.630$ $p_6 = 0.260$ $p_7 = 0.328$ $p_8 = 0.873$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	6.0 [5.0–7.0]	6.0 [5.3–7.5]	6.0 [5.0–6.0]	6 [5–7]	6.0 [5.0–6.0]	$p_4 = 0.762$ $p_5 = 0.863$ $p_6 = 0.657$ $p_7 = 0.707$ $p_8 = 0.946$
HAS-BLED (scores), Me [C25–C75]	3.0 [2.0–4.0]	3.0 [3.0–4.0]	3.0 [2.0–4.0]	3 [2.5–4]	3.0 [3.0–4.0]	$p_4 = 0.606$ $p_5 = 0.941$ $p_6 = 0.442$ $p_7 = 0.611$ $p_8 = 0.847$
Number of MPs (scores), Me [C25–C75]	6.0 [4.3–7.0]	7.0 [5.5–8.5]	7.0 [4.0–8.8]	6.0 [5.0–8.0]	7.0 [5.0–8.5]	$p_4 = 0.166$ $p_5 = 0.755$ $p_6 = 0.444$ $p_7 = 0.724$ $p_8 = –$
Number of MPs ≥ 5, abs. (%)	9/12 (75)	16/17 (94.1)	7/12 (58.3)	25/29 (86.2)	23/29 (79.3)	$p_4 = 0.141$ $p_5 = 0.386$ $p_6 = 0.019$ $p_7 = 0.05$ $p_8 = 0.762$
Dose of rivaroxaban 15 mg, abs. (%)	9/12 (75)	16/17 (94.1)	11/12 (91.7)	25/29 (86.2)	27/29 (93.1)	$p_4 = 0.141$ $p_5 = 0.273$ $p_6 = 0.798$ $p_7 = 0.627$ $p_8 = 0.107$
Dose of rivaroxaban 20 mg, abs. (%)	3/12 (25)	1/17 (5.9)	1/12 (8.3)	4/29 (13.8)	2/29 (6.9)	$p_4 = 0.141$ $p_5 = 0.273$ $p_6 = 0.798$ $p_7 = 0.627$ $p_8 = 0.107$
Creatinine (μmol/l), Me [C25–C75]	97.4 [80.5–121.8]	92.7 [79.7–120.5]	107 [94–137]	97.0 [79.7–121.5]	97.9 [87.9–120.5]	$p_4 = 0.744$ $p_5 = 0.378$ $p_6 = 0.097$ $p_7 = 0.127$ $p_8 = 0.810$
Hemoglobin (g/l), Me [C25–C75]	133.0 [94.0–138.0]	127.0 [119.5–132.0]	111 [106–119]	128.0 [119.0–133.8]	121.0 [111.0–130.0]	$p_4 = 0.547$ $p_5 = 0.211$ $p_6 = 0.007$ $p_7 = 0.016$ $p_8 = 0.308$
Platelets (10^9/l), Me [C25–C75]	239.0 [198.0–293.0]	239.0 [190.0–313.5]	221.0 [177.3–279.0]	239.0 [194.3–297.5]	226.0 [180.5–291.5]	$p_4 = 0.926$ $p_5 = 0.413$ $p_6 = 0.444$ $p_7 = 0.358$ $p_8 = 0.654$
Comorbidities						
CHD, abs. (%)	11/12 (91.7)	17/17 (100)	12/12 (100)	28/29 (96.6)	29/29 (100)	$p_4 = 0.226$ $p_5 = 0.307$ $p_6 = –$ $p_7 = 0.515$ $p_8 = 0.116$
Effort-induced angina pectoris, abs. (%)	8/12 (66.7)	10/17 (58.8)	9/12 (75)	18/29 (62.1)	19/29 (65.5)	$p_4 = 0.668$ $p_5 = 0.653$ $p_5 = 0.367$ $p_7 = 0.427$ $p_8 = 0.944$

TABLE 5 (*continued*)

Heart failure, abs. (%)	11/12 (91.7)	16/17 (94.1)	11/12 (91.7)	27/29 (93.1)	27/29 (93.1)	$p_4 = 0.798$ $p_5 = 1.000$ $p_6 = 0.798$ $p_7 = 0.872$ $p_8 = 0.872$
Arterial hypertension, abs. (%)	12/12 (100)	17/17 (100)	10/12 (83.3)	29/29 (100)	27/29 (93.1)	$p_4 = –$ $p_5 = 0.140$ $p_6 = 0.081$ $p_7 = 0.024$ $p_8 = 0.351$
History of previous myocardial infarction, abs. (%)	5/12 (41.7)	4/17 (23.5)	5/12 (41.7)	9/29 (31)	9/29 (31)	$p_4 = 0.298$ $p_5 = 1.000$ $p_6 = 0.298$ $p_7 = 0.514$ $p_8 = 0.514$
ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	0/12 (0)	6/17 (35.3)	3/12 (25)	6/29 (20.7)	9/29 (31)	$p_4 = 0.021$ $p_5 = 0.064$ $p_6 = 0.555$ $p_7 = 0.762$ $p_8 = 0.029$
Bronchial asthma, abs. (%)	1/12 (8.3)	1/17 (5.9)	3/12 (25)	2/29 (6.9)	4/29 (13.8)	$p_4 = 0.798$ $p_5 = 0.273$ $p_6 = 0.141$ $p_7 = 0.107$ $p_8 = 0.627$
Lower extremity atherosclerosis, abs. (%)	0/12 (0)	6/17 (35.3)	5/12 (41.7)	6/29 (20.7)	11/29 (37.9)	$p_4 = 0.021$ $p_5 = 0.012$ $p_6 = 0.728$ $p_7 = 0.168$ $p_8 = 0.013$
Chronic bronchitis, abs. (%)	5/12 (41.7)	3/17 (17.6)	3/12 (25)	8/29 (27.6)	6/29 (20.7)	$p_4 = 0.154$ $p_5 = 0.386$ $p_6 = 0.630$ $p_7 = 0.865$ $p_8 = 0.168$
Type 2 diabetes mellitus, abs. (%)	1/12 (8.3)	5/17 (29.4)	2/12 (16.7)	6/29 (20.7)	7/29 (24.1)	$p_4 = 0.168$ $p_5 = 0.537$ $p_6 = 0.430$ $p_7 = 0.767$ $p_8 = 0.245$
Charlson's comorbidity index (abs.), Me [C25-C75]	9.0 [9.0–10.0]	10.0 [9.0–11.5]	11.5 [10.0–13.8]	9 [9–11]	10.0 [9.0–12.0]	$p_4 = 0.471$ $p_5 = 0.008$ $p_5 = 0.048$ $p_7 = 0.01$ $p_8 = 0.083$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25-C75]	47.9 [39.0–59.4]	49.5 [35.3–55.5]	47.4 [39.3–52.5]	48.0 [37.1–57.6]	47.9 [37.4–53.7]	$p_4 = 1.000$ $p_5 = 0.843$ $p_6 = 0.948$ $p_7 = 0.877$ $p_8 = 0.899$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	0/12 (0)	1/17 (5.9)	1/12 (8.3)	1/29 (3.4)	2/29 (6.9)	$p_4 = 0.393$ $p_5 = 0.328$ $p_6 = 0.798$ $p_7 = 0.509$ $p_8 = 0.351$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	–	28.6	26.8	28.6	27.7 [26.8–27.7]	$p_4 = –$ $p_5 = –$ $p_6 = 1.000$ $p_7 = 1.000$ $p_8 = 0.351$
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	5/12 (41.7)	5/17 (29.4)	3/12 (25)	10/29 (34.5)	8/29 (27.6)	$p_4 = 0.494$ $p_5 = 0.304$ $p_6 = 0.793$ $p_7 = 0.553$ $p_8 = 0.378$

TABLE 5 (*continued*)

GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	38.6 [34.5–41.7]	35.1 [33.1–38.3]	38.4 [36.4–38.4]	35.3 [34.5–40.5]	35.9 [34.7–40.4]	$p_4 = 0.548$ $p_5 = 1.000$ $p_6 = 0.143$ $p_7 = 0.371$ $p_8 = 0.378$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	5/12 (41.7)	9/17 (52.9)	7/12 (58.3)	14/29 (48.3)	16/29 (55.2)	$p_4 = 0.550$ $p_5 = 0.292$ $p_6 = 0.774$ $p_7 = 0.558$ $p_8 = 0.431$
GFR in CKD Stage 3A (ml/min/1.73 ^{2}), Me [C25–C75]	48.2 [47.9–59.3]	51.3 [47.9–54.1]	51.9 [46.8–52.5]	51.2 [47.9–55.5]	51.5 [47.5–54.3]	$p_4 = 0.833$ $p_5 = 0.530$ $p_5 = 1.000$ $p_7 = 0.699$ $p_8 = 0.431$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	2/12 (16.7)	2/17 (11.8)	1/12 (8.3)	4/29 (13.8)	3/29 (10.3)	$p_4 = 0.706$ $p_5 = 0.484$ $p_6 = 0.765$ $p_7 = 0.627$ $p_8 = 0.574$
GFR in CKD Stage 2 (ml/min/1.73 m^2), Me [C25–C75]	61.6 [61.1–61.6]	65.1 [59.7–65.1]	64.19	62.1 [60.4–65.3]	65.1 [64.2–65.1]	$p_4 = 0.800$ $p_5 = 0.667$ $p_6 = 1.000$ $p_7 = 1.000$ $p_8 = 0.574$
CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	–	–	–	–	–	–
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	–	–	–	–	–	–

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

TABLE 6

PECULIARITIES OF DRUG INTERACTION IN PATIENTS-CARRIERS OF TT GENOTYPE OF *ABCB1* GENE (rs1045642)

Indicators	TT genotype (n = 41) of the *ABCB1* gene (rs1045642)			*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	12/41 (29.3)	17/41 (41.4)	12/41 (29.3)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	57.5 [36.3–91.2]	63.5 [33.5–114.5]	71.2 [33.7–89.1]	$p_4 = 0.859$ $p_5 = 0.908$ $p_6 = 0.912$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	3.6 [2.2–5.7]	4.2 [2.2–7.1]	4.6 [2.3–5.4]	$p_4 = 0.690$ $p_5 = 0.603$ $p_6 = 0.912$
Prothrombin time (s), Me [C25–C75]	14.1 [13.5–14.5]	14.0 [12.4–15.8]	15.9 [13.4–17.8]	$p_4 = 0.842$ $p_5 = 0.126$ $p_6 = 0.066$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	1/12 (8.3)	2/17 (11.8)	9/12 (75)	$p_4 = 0.765$ $p_5 = 0.001$ $p_6 = 0.001$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

Group 1. Wild-type (CC) carriers of the *ABCB1* gene (rs4148738)

We divided group 1 patients (n = 37) into subgroups cause-specific to concomitant therapy. Subgroup 1 – 14 patients taking rivaroxaban without CCB (median age – 88.0 [83.3–91.0] years; 71.4 % women); subgroup 2 – 14 patients taking rivaroxaban + amlodipine (median age – 88.0 [82.8–90.3] years; 85.7 % women); subgroup 3 – 9 patients taking rivaroxaban + verapamil (median age – 89.0 [81.0–92.0] years; 66.7 % women). The baseline characterization of patients according to rs4148738 (*ABCB1*) genotype is presented in Table 7. The patients in the study groups were comparable with respect to the main parameters.

Among wild-type (CC) carriers of the *ABCB1* gene (rs4148738), the $C_{min, ss}$ level of rivaroxaban was statistically significantly higher in the group of patients taking rivaroxaban + verapamil compared with the group of patients taking rivaroxaban without CCB (Me 77.6 [47.4–115.3] vs. 29.4 [14.5–61.9] ng/ml; $p = 0.014$). The $C_{min, ss}$/D levels of rivaroxaban were higher in the group of patients receiving rivaroxaban + verapamil than in the group of patients not administering CCB in combination with rivaroxaban, but without reaching statistical significance (Me 5.2 [2.3–7.7] vs. 2.0 [1.0–3.8] ng/ml/mg; $p = 0.020$). No statistically significant differences were found in the PT rate and the number of patients who experienced AR in the form of clinically significant small bleedings between the compared groups ($p > 0.017$) (Table 8).

Group 2. Heterozygous type (CT) carriers of the *ABCB1* gene (rs4148738)

We divided group 2 patients (n = 63) into subgroups cause-specific to concomitant therapy. Subgroup 1 – 24 patients taking rivaroxaban without CCB (median age – 88.0 [83.0–91.0] years; 70.8 % women); subgroup 2 – 27 patients taking rivaroxaban + amlodipine (median age – 86.0 [83.0–88.0] years; 81.5 % women); subgroup 3 –

TABLE 7

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE CC GENOTYPE OF THE *ABCB1* GENE (rs4148738)

Indicators	CC genotype (n = 37) of the *ABCB1* gene (rs4148738)					
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	p value
Number of patients, abs. (%)	14/37 (37.85)	14/37 (37.85)	9/37 (24.3)	28/37 (75.7)	23/37 (62.2)	–
Age (years), Me [C25–C75]	88.0 [83.3–91.0]	88.0 [82.8–90.3]	89.0 [81.0–92.0]	88.0 [83.3–90.75]	88.0 [82.0–91.0]	$p_4 = 0.982$ $p_5 = 0.975$ $p_6 = 0.926$ $p_7 = 0.931$ $p_8 = 0.963$
Women, abs. (%)	10/14 (71.4)	12/14 (85.7)	6/9 (66.7)	22/28 (78.6)	18/23 (78.3)	$p_4 = 0.357$ $p_5 = 0.809$ $p_6 = 0.280$ $p_7 = 0.469$ $p_8 = 0.639$
BMI (kg/m^2), Me [C25–C75]	29.1 [25.3–30.7]	28.6 [25.6–31.9]	28.6 [23.6–30.9]	29.1 [25.4–31.2]	28.6 [25.6–31.5]	$p_4 = 0.894$ $p_5 = 0.804$ $p_6 = 0.851$ $p_7 = 0.789$ $p_8 = 0.957$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	4.5 [3.0–5.8]	6.0 [5.3–6.0]	6.0 [5.0–8.5]	5.0 [4.0–6.0]	6.0 [5.5–6.5]	$p_4 = 0.057$ $p_5 = 0.064$ $p_6 = 0.524$ $p_7 = 0.129$ $p_8 = 0.019$
HAS-BLED (scores), Me [C25–C75]	4.0 [2.3–5.0]	3.0 [3.0–3.0]	4.0 [2.0–5.0]	3.0 [3.0–4.8]	3.0 [3.0–4.0]	$p_4 = 0.208$ $p_5 = 0.799$ $p_6 = 0.284$ $p_7 = 0.767$ $p_8 = 0.295$
Number of MPs (scores), Me [C25–C75]	6.0 [5.0–7.3]	7.0 [5.0–9.0]	6.0 [4.5–8.0]	6.0 [5.0–8.8]	6.0 [5.0–8.0]	$p_4 = 0.285$ $p_5 = 0.877$ $p_6 = 0.277$ $p_7 = 0.614$ $p_8 = 0.429$

TABLE 7 (*continued*)

Number of MPs ≥ 5, abs. (%)	12/14 (85.7)	14/14 (100)	7/9 (77.8)	26/28 (92.9)	21/23 (91.3)	$p_4 = 0.142$ $p_5 = 0.624$ $p_6 = 0.065$ $p_7 = 0.205$ $p_8 = 0.595$
Dose of rivaroxaban 15 mg, abs. (%)	12/14 (85.7)	11/14 (78.6)	8/9 (88.9)	23/28 (82.1)	19/23 (82.6)	$p_4 = 0.622$ $p_5 = 0.825$ $p_6 = 0.524$ $p_7 = 0.633$ $p_8 = 0.804$
Dose of rivaroxaban 20 mg, abs. (%)	2/14 (14.3)	3/14 (21.4)	1/9 (11.1)	5/28 (17.9)	4/23 (17.4)	$p_4 = 0.622$ $p_5 = 0.825$ $p_6 = 0.524$ $p_7 = 0.633$ $p_8 = 0.804$
Creatinine (μmol/l), Me [C25–C75]	106.5 [95.9–127.5]	99.2 [93.6–152.5]	102.0 [92.7–118.0]	121.0 [112.5–129.8]	99.2 [93.6–126.5]	$p_4 = 0.839$ $p_5 = 0.643$ $p_6 = 0.829$ $p_7 = 0.689$ $p_8 = 0.689$
Hemoglobin (g/l), Me [C25–C75]	123.5 [114.8–140.3]	121.0 [104.8–125.5]	127.0 [119.0–133.6]	121.0 [112.5–130.8]	124.0 [112.0–128.0]	$p_4 = 0.246$ $p_5 = 1.000$ $p_6 = 0.159$ $p_7 = 0.453$ $p_8 = 0.411$
Platelets (10^9/l), Me [C25–C75]	247 [188.5–264.5]	239.5 [195.8–328.3]	202.0 [170.0–344.5]	246.0 [193.0–270.5]	220.0 [190.0–328.0]	$p_4 = 0.401$ $p_5 = 0.926$ $p_6 = 0.336$ $p_7 = 0.542$ $p_8 = 0.610$
Comorbidities						
CHD, abs. (%)	14/14 (100)	14/14 (100)	9/9 (100)	28/28 (100)	23/23 (100)	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
Effort-induced angina pectoris, abs. (%)	12/14 (85.7)	13/14 (92.9)	8/9 (88.9)	25/28 (89.3)	21/23 (91.3)	$p_4 = 0.541$ $p_5 = 0.825$ $p_6 = 0.742$ $p_7 = 0.973$ $p_8 = 0.595$
Heart failure, abs. (%)	14/14 (100)	13/14 (92.9)	8/9 (88.9)	27/28 (96.4)	21/23 (91.3)	$p_4 = 0.309$ $p_5 = 0.202$ $p_6 = 0.742$ $p_7 = 0.384$ $p_8 = 0.257$
Arterial hypertension, abs. (%)	13/14 (92.9)	13/14 (92.9)	8/9 (88.9)	26/28 (92.9)	21/23 (91.3)	$p_4 = 1.000$ $p_5 = 0.742$ $p_6 = 0.742$ $p_7 = 0.704$ $p_8 = 0.867$
History of previous myocardial infarction, abs. (%)	1/14 (7.1)	4/14 (28.6)	3/9 (33.3)	5/28 (17.9)	7/23 (30.4)	$p_4 = 0.139$ $p_5 = 0.106$ $p_6 = 0.809$ $p_7 = 0.327$ $p_8 = 0.095$
ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	0/14 (0)	4/14 (28.6)	3/9 (33.3)	4/28 (14.3)	7/23 (30.4)	$p_4 = 0.031$ $p_5 = 0.021$ $p_6 = 0.809$ $p_7 = 0.204$ $p_8 = 0.022$
Bronchial asthma, abs. (%)	0/14 (0)	1/14 (7.1)	2/9 (22.2)	1/28 (3.6)	3/23 (13)	$p_4 = 0.309$ $p_5 = 0.064$ $p_6 = 0.295$ $p_7 = 0.075$ $p_8 = 0.159$

TABLE 7 (*continued*)

Lower extremity atherosclerosis, abs. (%)	0/14 (0)	2/14 (14.3)	1/9 (11.1)	2/28 (7.1)	3/23 (13)	$p_4 = 0.142$ $p_5 = 0.202$ $p_6 = 0.825$ $p_7 = 0.704$ $p_8 = 0.159$
Chronic bronchitis, abs. (%)	5/14 (35.7)	4/14 (28.6)	4/9 (44.4)	9/28 (32.1)	8/23 (24.8)	$p_4 = 0.686$ $p_5 = 0.675$ $p_6 = 0.435$ $p_7 = 0.501$ $p_8 = 0.954$
Type 2 diabetes mellitus, abs. (%)	5/14 (35.7)	5/14 (35.7)	0/9 (0)	10/28 (35.7)	5/23 (21.7)	$p_4 = 1.000$ $p_5 = 0.043$ $p_6 = 0.043$ $p_7 = 0.036$ $p_8 = 0.353$
Charlson's comorbidity index (abs.), Me [C25-C75]	10.5 [9.0–11.0]	10.0 [9.0–12.0]	11.0 [9.0–12.5]	10.0 [9.0–11.0)]	10.0 [9.0–12.0]	$p_4 = 0.667$ $p_5 = 0.403$ $p_6 = 0.734$ $p_7 = 0.519$ $p_8 = 0.467$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25-C75]	41.2 [36.5–49.3]	43.3 [33.1–47.5]	44.6 [35.7–48.2]	42.2 [36.2–45.9]	44.1 [35.7–48.0]	$p_4 = 0.910$ $p_5 = 0.877$ $p_6 = 0.734$ $p_7 = 0.768$ $p_8 = 0.962$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	1/14 (7.1)	3/14 (21.4)	0/9 (0)	4/28 (14.3)	3/23 (13.0)	$p_4 = 0.280$ $p_5 = 0.412$ $p_6 = 0.136$ $p_7 = 0.230$ $p_8 = 0.546$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	29.3	21.4 [19.5–21.4]	0	23.0 [19.9–28.3]	21.4 [19.5–21.4]	$p_4 = 0.500$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = 0.500$
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	9/14 (64.3)	6/14 (42.9)	4/9 (44.4)	15/28 (53.6)	10/23 (43.5)	$p_4 = 0.256$ $p_5 = 0.349$ $p_6 = 0.940$ $p_7 = 0.634$ $p_8 = 0.270$
GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	38.7 [36.3–42.7]	40.8 [36.5–44.1]	35.7 [34.3–37.2]	39.0 [36.7–43.5]	37.1 [35.7–44.1]	$p_4 = 0.456$ $p_5 = 0.106$ $p_6 = 0.067$ $p_7 = 0.049$ $p_8 = 1.000$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	1/14 (7.1)	3/14 (21.4)	4/9 (44.4)	4/28 (14.3)	7/23 (30.4)	$p_4 = 0.280$ $p_5 = 0.034$ $p_6 = 0.242$ $p_7 = 0.056$ $p_8 = 0.236$
GFR in CKD Stage 3A (ml/min/1.73 m^2), Me [C25–C75]	45.8	46.1 [45.5–46.1]	46.5 [44.7–48.4]	45.9 [45.6–50.3]	47.9 [45.5–51.6]	$p_4 = 1.000$ $p_5 = 1.000$ $p_6 = 0.629$ $p_7 = 0.686$ $p_8 = 0.667$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	2/14 (14.3)	2/14 (14.3)	1/9 (11.1)	4/28 (14.3)	2/23 (8.7)	$p_4 = 1.000$ $p_5 = 0.825$ $p_6 = 0.825$ $p_7 = 0.809$ $p_8 = 0.837$
GFR in CKD Stage 2 (ml/min/1.73 ^{2}), Me [C25–C75]	61.1 [59.9–61.1]	70.4 [59.7–70.4]	85.97	61.1 [59.8–76.4]	83.5 [81.1–83.5]	$p_4 = 1.000$ $p_5 = 0.667$ $p_6 = 0.667$ $p_7 = 0.400$ $p_8 = 0.667$

TABLE 7 (*continued*)

CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	1/14 (7.1)	0/14 (0)	0/9 (0)	1/28 (3.6)	–	$p_4 = 0.309$ $p_5 = 0.412$ $p_6 = –$ $p_7 = 0.565$ $p_8 = 0.204$
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	96.9	0	0	96.96	–	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

TABLE 8

FEATURES OF DRUG INTERACTION IN PATIENTS CARRYING THE CC GENOTYPE OF THE *ABCB1* GENE (rs4148738)

Indicators	CC genotype (*n* = 37) of the *ABCB1* gene (rs4148738)			*p value*
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	14/37 (37.85)	14/37 (37.85)	9/37 (24.3)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	29.4 [14.5–61.9]	71.2 [28.3–102.8]	77.6 [47.4–115.3]	$p_4 = 0.081$ $p_5 = 0.014$ $p_6 = 0.571$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	2.0 [1.0–3.8]	4.6 [1.9–6.9]	5.2 [2.3–7.7]	$p_4 = 0.098$ $p_5 = 0.020$ $p_6 = 0.378$
Prothrombin time (s), Me [C25–C75]	12.7 [12.3–14.2]	13.3 [12.2–14.7]	13.5 [13.1–15.6]	$p_4 = 0.581$ $p_5 = 0.243$ $p_6 = 0.488$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	1/14 (7.1)	1/14 (7.1)	1/9 (11.1)	$p_4 = 1.000$ $p_5 = 0.742$ $p_6 = 0.742$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

12 patients taking rivaroxaban + verapamil (median age – 88.5 [81.3–90.0] years; 75.0 % women). The baseline characterization of patients according to rs4148738 (*ABCB1*) genotype is presented in Table 9. The patients in the study groups were comparable with respect to the main parameters.

Among carriers of the heterozygous type (CT) of the *ABCB1* gene (rs4148738), the levels of $C_{min, ss}$ of rivaroxaban and $C_{min, ss}$/D of rivaroxaban were higher in the group of patients taking rivaroxaban + verapamil and rivaroxaban + amlodipine than in the group of patients not taking CCB in combination with rivaroxaban, but these differences did not reach statistical significance (Table 10). PT was higher in the group of patients taking rivaroxaban + verapamil than in the group of patients not taking CCB in combination with rivaroxaban, but these differences also did not reach statistical significance (14.6 [13.5–16.8] vs. 14.0 [12.8–14.5] s; $p = 0.083$) (Table 10). No differences in the number of patients who experienced AR in the form of clinically significant small hemorrhages were found between the compared groups ($p > 0.017$) (Table 10).

Group 3. Homozygous type (TT) carriers of the *ABCB1* gene (rs4148738)

We divided patients in group 3 ($n = 28$) into subgroups cause-specific to concomitant therapy. Subgroup 1 – 9 patients taking rivaroxaban without CCB (median age – 88.0 [84.0–89.5] years; 77.8 % women); subgroup 2 – 10 patients taking rivaroxaban + amlodipine (median age – 84.0 [81.8–89.3] years; 100 % women); subgroup 3 – 9 patients taking rivaroxaban + verapamil (median age – 88.0 [86.0–92.0] years; 33.3 % women). The baseline characterization of patients according to rs4148738 *(ABCB1)* gen-

TABLE 9

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE CT GENOTYPE OF THE *ABCB1* GENE (rs4148738)

Indicators	CT genotype (n = 63) of the *ABCB1* gene (rs4148738)					p value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	
Number of patients, abs. (%)	24/63 (38.1)	27/63 (42.9)	12/63 (19)	51/63 (81)	39/63 (61.9)	–
Age (years), Me [C25–C75]	88.0 [83.0–91.0]	86.0 [83.0–88.0]	88.5 [81.3–90.0]	87.0 [83.0–90.0]	87.0 [82.0–89.0]	$p_4 = 0.320$ $p_5 = 0.908$ $p_6 = 0.391$ $p_7 = 0.661$ $p_8 = 0.756$
Women, abs. (%)	17/24 (70.8)	22/27 (81.5)	9/12 (75.0)	39/51 (76.5)	31/39 (79.5)	$p_4 = 0.371$ $p_5 = 0.792$ $p_6 = 0.644$ $p_7 = 0.914$ $p_8 = 0.434$
BMI (kg/m^2), Me [C25–C75]	28.5 [25.5–31.1]	30.1 [25.6–31.4]	31.3 [24.1–32.8]	29.0 [25.6–31.2]	30.4 [25.0–31.8]	$p_4 = 0.695$ $p_5 = 0.440$ $p_6 = 0.786$ $p_7 = 0.554$ $p_8 = 0.523$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	6 [5–7]	6.0 [5.0–8.0]	5.5 [5.0–8.0]	6.0 [5.0–7.0]	6.0 [5.0–8.0]	$p_4 = 0.669$ $p_5 = 0.914$ $p_6 = 0.603$ $p_7 = 0.715$ $p_8 = 0.789$
HAS-BLED (scores), Me [C25–C75]	3 [2–3]	3.0 [3.0–3.75]	3.0 [2.75–4.0]	3.0 [3.0–3.0]	3.0 [3.0–4.0]	$p_4 = 0.708$ $p_5 = 0.604$ $p_6 = 0.812$ $p_7 = 0.669$ $p_8 = 0.568$
Number of MPs (scores), Me [C25–C75]	6 [5–7]	7.0 [6.0–9.0]	6.0 [5.25–7.0]	6.0 [5.0–8.0]	7.0 [6.0–8.0]	$p_4 = 0.019$ $p_5 = 0.753$ $p_6 = 0.080$ $p_7 = 0.396$ $p_8 = 0.056$
Number of MPs ≥ 5, abs. (%)	20/24 (83.3)	25/27 (92.6)	10/12 (83.3)	45/51 (88.2)	35/39 (89.7)	$p_4 = 0.306$ $p_5 = 1.000$ $p_6 = 0.379$ $p_7 = 0.646$ $p_8 = 0.244$
Dose of rivaroxaban 15 mg, abs. (%)	21/24 (87.5)	24/27 (88.9)	11/12 (91.7)	45/51 (88.2)	35/39 (89.7)	$p_4 = 0.878$ $p_5 = 0.708$ $p_6 = 0.792$ $p_7 = 0.734$ $p_8 = 0.783$
Dose of rivaroxaban 20 mg, abs. (%)	3/24 (12.5)	3/27 (11.1)	1/12 (8.3)	6/51 (11.8)	3/39 (7.7)	$p_4 = 0.878$ $p_5 = 0.708$ $p_6 = 0.792$ $p_7 = 0.734$ $p_8 = 0.783$
Creatinine (μmol/l), Me [C25–C75]	104.0 [89.5–124.3]	97.9 [83.6–122.0]	92.3 [83.6–101.5]	98.1 [86.0–122.0]	94.7 [83.6–110.0]	$p_4 = 0.336$ $p_5 = 0.146$ $p_6 = 0.578$ $p_7 = 0.270$ $p_8 = 0.179$

TABLE 9 (*continued*)

Hemoglobin (g/l), Me [C25–C75]	123.0 [110.3–136.0]	125.0 [113.0–129.0]	124.5 [101.5–132.3]	125.0 [112.0–132.0]	125.0 [112.0–130.0]	$p_4 = 0.799$ $p_5 = 0.497$ $p_6 = 0.869$ $p_7 = 0.649$ $p_8 = 0.630$
Platelets (10^9/l), Me [C25–C75]	215.0 [160.5–259.0]	236.0 [190.0–305.0]	194.5 [161.3–260.5]	224.0 [175.0–276.0]	222.0 [178.0–293.0]	$p_4 = 0.122$ $p_5 = 0.987$ $p_6 = 0.168$ $p_7 = 0.436$ $p_8 = 0.240$
Comorbidities						
CHD, abs. (%)	24/24 (100)	27/27 (100)	12/12 (100)	51/51 (100)	39/39 (100)	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
Effort-induced angina pectoris, abs. (%)	21/24 (87.5)	20/27 (74.1)	9/12 (75)	41/51 (80.4)	29/39 (74.4)	$p_4 = 0.228$ $p_5 = 0.343$ $p_6 = 0.951$ $p_7 = 0.678$ $p_8 = 0.211$
Heart failure, abs. (%)	23/24 (95.8)	25/27 (92.6)	10/12 (83.3)	48/51 (94.1)	35/39 (89.7)	$p_4 = 0.623$ $p_5 = 0.201$ $p_6 = 0.379$ $p_7 = 0.214$ $p_8 = 0.385$
Arterial hypertension, abs. (%)	23/24 (95.8)	26/27 (96.3)	11/12 (91.7)	49/51 (96.1)	37/39 (94.9)	$p_4 = 0.932$ $p_5 = 0.607$ $p_6 = 0.545$ $p_7 = 0.518$ $p_8 = 0.862$
History of previous myocardial infarction, abs. (%)	9/24 (37.5)	6/27 (22.2)	3/12 (25)	15/51 (29.4)	9/39 (23.1)	$p_4 = 0.232$ $p_5 = 0.453$ $p_6 = 0.849$ $p_7 = 0.761$ $p_8 = 0.218$
ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	6/24 (25.0)	6/27 (22.2)	3/12 (25)	12/51 (23.5)	9/39 (23.1)	$p_4 = 0.815$ $p_5 = 1.000$ $p_6 = 0.849$ $p_7 = 0.914$ $p_8 = 0.862$
Bronchial asthma, abs. (%)	0/24 (0)	0/27 (0)	2/12 (16.7)	0/51 (0)	2/39 (5.1)	$p_4 = –$ $p_5 = 0.040$ $p_6 = 0.029$ $p_7 = 0.003$ $p_8 = 0.260$
Lower extremity atherosclerosis, abs. (%)	3/24 (12.5)	7/27 (25.9)	4/12 (33.3)	10/51 (19.6)	11/39 (28.2)	$p_4 = 0.228$ $p_5 = 0.137$ $p_6 = 0.635$ $p_7 = 0.303$ $p_8 = 0.145$
Chronic bronchitis, abs. (%)	11/24 (45.8)	9/27 (33.3)	5/12 (41.7)	20/51 (39.2)	14/39 (35.9)	$p_4 = 0.361$ $p_5 = 0.813$ $p_6 = 0.617$ $p_7 = 0.876$ $p_8 = 0.434$
Type 2 diabetes mellitus, abs. (%)	7/24 (29.2)	7/27 (25.9)	3/12 (25)	14/51 (27.5)	10/39 (25.6)	$p_4 = 0.796$ $p_5 = 0.792$ $p_6 = 0.951$ $p_7 = 0.863$ $p_8 = 0.759$

TABLE 9 (*continued*)

Charlson's comorbidity index (abs.), Me [C25–C75]	10.0 [9.3–11.0]	10.0 [9.0–11.0]	10.0 [9.0–11.0]	10.0 [9.0–11.0]	10.0 [9.0–11.0]	$p_4 = 0.369$ $p_5 = 0.631$ $p_6 = 0.916$ $p_7 = 0.844$ $p_8 = 0.240$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25–C75]	41.2 [36.5–49.3]	43.3 [33.1–47.5]	44.6 [35.7–48.2]	42.2 [36.2–45.9]	51.8 [40.5–56.0]	$p_4 = 0.910$ $p_5 = 0.877$ $p_6 = 0.734$ $p_7 = 0.768$ $p_8 = 0.374$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	2/24 (8.3)	3/27 (11.1)	1/12 (8.3)	5/51 (9.8)	4/39 (10.3)	$p_4 = 0.739$ $p_5 = 1.000$ $p_6 = 0.792$ $p_7 = 0.876$ $p_8 = 0.801$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	29.3	21.4 [19.5–21.4]	0	23.0 [19.9–28.3]	28.9 [26.9–29.5]	$p_4 = 0.500$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = 0.800$
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	8/24 (33.3)	9/27 (33.3)	1/12 (8.3)	17/51 (33.3)	10/39 (25.6)	$p_4 = 1.000$ $p_5 = 0.102$ $p_6 = 0.099$ $p_7 = 0.085$ $p_8 = 0.512$
GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	38.7 [36.3–42.7]	40.8 [36.5–44.1]	35.7 [34.3–37.2]	39.0 [36.7–43.5]	38.0 [34.2–41.9]	$p_4 = 0.456$ $p_5 = 0.106$ $p_6 = 0.067$ $p_7 = 0.049$ $p_8 = 0.897$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	13/24 (54.2)	11/27 (40.7)	8/12 (66.7)	24/51 (47.1)	19/39 (48.7)	$p_4 = 0.338$ $p_5 = 0.473$ $p_6 = 0.135$ $p_7 = 0.222$ $p_8 = 0.674$
GFR in CKD Stage 3A (ml/min/1.73 m^2), Me [C25–C75]	45.8	46.1 [45.5–46.1]	46.5 [44.7–48.4]	45.9 [45.6–50.3]	53.9 [50.5–55.8]	$p_4 = 1.000$ $p_5 = 1.000$ $p_6 = 0.629$ $p_7 = 0.686$ $p_8 = 0.077$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	1/24 (4.2)	4/27 (14.8)	2/12 (16.7)	5/51 (9.8)	6/39 (15.4)	$p_4 = 0.202$ $p_5 = 0.201$ $p_6 = 0.882$ $p_7 = 0.496$ $p_8 = 0.169$
GFR in CKD Stage 2 (ml/min/1.73 m^2), Me [C25–C75]	61.1 [59.9–61.1]	70.4 [59.7–70.4]	85.97	61.1 [59.8–76.4]	66.1 [63.4–69.2]	$p_4 = 1.000$ $p_5 = 0.667$ $p_6 = 0.667$ $p_7 = 0.400$ $p_8 = 0.286$
CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	–	–	–	–	–	–
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	96.6	96.9	0	0		$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

otype is presented in Table 11. Patients in subgroup 3 (rivaroxaban + verapamil) had a higher proportion of women, had higher creatinine levels and lower haemoglobin levels than those in subgroup 2 (rivaroxaban + amlodipine), and had a higher Charlson comorbidity index than patients in subgroup 1 (rivaroxaban without CCB). In other parameters, patients in the studied subgroups were comparable.

Among homozygous type (TT) carriers of the *ABCB1* gene (rs4148738), the levels of $C_{min, ss}$ of rivaroxaban, $C_{min, ss}$/D of rivaroxaban and PT were higher in patients taking rivaroxaban + verapamil compared to all study groups, but without reaching statistical significance of the differences. AR in the form of clinically significant minor bleeding occurred quite frequently – in 77.8 % of cases in the group of patients taking rivaroxaban + verapamil, and this rate was statistically significantly higher in comparison with the group of patients not taking CCB in combination with rivaroxaban (11.1 %; $p = 0.004$), and in comparison with the group of patients taking rivaroxaban + amlodipine, the difference reached statistical significance (30 %; $p = 0.037$) (Table 12).

DISCUSSION

The data obtained reveal that in patients 80 years and older with non-valvular AF and carriers of the wild-type genotype (CC) of the *ABCB1* gene (rs1045642), co-administration of rivaroxaban with CCBs (amlodipine or verapamil), which are capable of drug interaction with rivaroxaban, resulted in higher values of $C_{min, ss}$ of rivaroxaban compared with patients not taking CCBs. In carriers of heterozygous genotype (CT) of *ABCB1* gene (rs1045642), co-

TABLE 10

FEATURES OF DRUG INTERACTION IN PATIENTS CARRYING THE CT GENOTYPE OF THE *ABCB1* GENE (rs4148738)

Indicators	CT genotype ($n = 63$) of the *ABCB1* gene (rs4148738)			*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	24/63 (38.1)	27/63 (42.9)	12/63 (19)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	41.7 [25.4–58.2]	56.4 [29.3–91.3]	56.0 [34.1–114.2]	$p_4 = 0.093$ $p_5 = 0.112$ $p_6 = 0.738$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	2.6 [1.7–3.4]	3.8 [2.0–4.6]	3.7 [2.1–7.6]	$p_4 = 0.093$ $p_5 = 0.131$ $p_6 = 0.715$
Prothrombin time (s), Me [C25–C75]	14.0 [12.7–14.5]	13.3 [12.6–14.6]	14.6 [13.5–16.8]	$p_4 = 0.962$ $p_5 = 0.083$ $p_6 = 0.117$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	4/24 (16.7)	3/27 (11.1)	2/12 (16.7)	$p_4 = 0.565$ $p_5 = 1.000$ $p_6 = 0.632$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

TABLE 11

BASELINE CHARACTERISTICS OF PATIENTS CARRYING THE TT GENOTYPE OF THE *ABCB1* GENE (rs4148738)

Indicators	TT genotype ($n = 28$) of the *ABCB1* gene (rs4148738)					*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	Rivaroxaban without CCB and rivaroxaban + amlodipine	Rivaroxaban and amlodipine and rivaroxaban + verapamil	
Number of patients, abs. (%)	9/28 (32.1)	10/28 (35.8)	9/28 (32.1)	19/28 (67.9)	19/28 (67.9)	–
Age (years), Me [C25–C75]	88.0 [84.0–89.5]	84.0 [81.8–89.3]	88.0 [86.0–92.0]	86.0 [82.0–89.0]	87.0 [83.0–90.0]	$p_4 = 0.400$ $p_5 = 0.666$ $p_6 = 0.113$ $p_7 = 0.243$ $p_8 = 0.809$

TABLE 11 (*continued*)

Women, abs. (%)	7/9 (77.8)	10/10 (100)	3/9 (33.3)	17/19 (89.5)	13/19 (68.4)	$p_4 = 0.115$ $p_5 = 0.058$ $p_6 = 0.002$ $p_7 = 0.002$ $p_8 = 0.609$
BMI (kg/m^2), Me [C25–C75]	25.6 [23.9–32.6]	27.0 [23.8–33.7]	26.8 [25.0–30.1]	26.9 [24.2–33.1]	26.9 [24.7–31.1]	$p_4 = 0.968$ $p_5 = 0.931$ $p_6 = 0.604$ $p_7 = 0.699$ $p_8 = 1.000$
Scale CHA_2DS_2-VASc (scores), Me [C25–C75]	6.0 [5.0–7.0]	6.0 [4.0–6.5]	6.0 [5.3–7.0]	6.0 [5.0–8.0]	6.0 [5.0–7.0]	$p_4 = 0.639$ $p_5 = 0.694$ $p_6 = 0.435$ $p_7 = 0.473$ $p_8 = 1.000$
HAS-BLED (scores), Me [C25–C75]	3.0 [1.75–4.5]	3.0 [3.0–4.5]	3.0 [2.0–4.0]	3.0 [3.0–4.0]	3.0 [5.0–7.0]	$p_4 = 0.429$ $p_5 = 1.000$ $p_6 = 0.222$ $p_7 = 0.442$ $p_8 = 0.765$
Number of MPs (scores), Me [C25–C75]	6.0 [4.0–8.0]	7.0 [6.0–8.0]	6.0 [4.0–9.0]	6.0 [5.0–8.0]	7.0 [6.0–8.0]	$p_4 = 0.243$ $p_5 = 0.666$ $p_6 = 0.842$ $p_7 = 0.923$ $p_8 = 0.332$
Number of MPs ≥ 5, abs. (%)	6/9 (66.7)	10/10 (100)	6/9 (66.7)	16/19 (84.2)	16/19 (84.2)	$p_4 = 0.047$ $p_5 = 1.000$ $p_6 = 0.047$ $p_7 = 0.291$ $p_8 = 0.291$
Dose of rivaroxaban 15 mg, abs. (%)	7/9 (77.8)	9/10 (90)	8/9 (88.9)	16/19 (84.2)	17/19 (89.5)	$p_4 = 0.466$ $p_5 = 0.527$ $p_6 = 0.937$ $p_7 = 0.741$ $p_8 = 0.409$
Dose of rivaroxaban 20 mg, abs. (%)	2/9 (22.2)	1/10 (10)	1/9 (11.1)	3/19 (15.8)	2/19 (10.5)	$p_4 = 0.466$ $p_5 = 0.527$ $p_6 = 0.937$ $p_7 = 0.741$ $p_8 = 0.409$
Creatinine (μmol/l), Me [C25–C75]	97.0 [84.9–117.5]	89.7 [85.0–95.5]	112 [98.6–146.5]	92.9 [85.6–97.7]	94.8 [86.9–112.0]	$p_4 = 0.156$ $p_5 = 0.161$ $p_6 = 0.006$ $p_7 = 0.014$ $p_8 = 0.962$
Hemoglobin (g/l), Me [C25–C75]	132.5 [98.0–144.3]	132.5 [117.8–138.0]	110 [106–117]	132.5 [115.5–138.0]	119.0 [110.0–136.0]	$p_4 = 0.762$ $p_5 = 0.277$ $p_6 = 0.01$ $p_7 = 0.027$ $p_8 = 0.696$
Platelets (10^9/l), Me [C25–C75]	264.5 [201.3–296.8]	208 [172.0–275.0]	216 [165–256]	215.0 [189.3–290.8]	213.0 [175.0–264.0]	$p_4 = 0.146$ $p_5 = 0.093$ $p_6 = 0.905$ $p_7 = 0.322$ $p_8 = 0.066$
Comorbidities						
CHD, abs. (%)	8/9 (88.9)	10/10 (100)	9/9 (100)	18/19 (94.7)	19/19 (100)	$p_4 = 0.279$ $p_5 = 0.303$ $p_6 = –$ $p_7 = 0.483$ $p_8 = 0.139$
Effort-induced angina pectoris, abs. (%)	5/9 (55.6)	7/10 (70)	8/9 (88.9)	12/19 (63.2)	15/19 (78.9)	$p_4 = 0.515$ $p_5 = 0.114$ $p_6 = 0.313$ $p_7 = 0.159$ $p_8 = 0.201$

TABLE 11 (*continued*)

Heart failure, abs. (%)	8/9 (88.9)	10/10 (100)	9/9 (100)	18/19 (94.7)	19/19 (100)	$p_4 = 0.279$ $p_5 = 0.303$ $p_6 = –$ $p_7 = 0.483$ $p_8 = 0.139$
Arterial hypertension, abs. (%)	9/9 (100)	9/10 (90)	8/9 (88.9)	18/19 (94.7)	17/19 (89.5)	$p_4 = 0.330$ $p_5 = 0.303$ $p_6 = 0.937$ $p_7 = 0.575$ $p_8 = 0.312$
History of previous myocardial infarction, abs. (%)	2/9 (22.2)	4/10 (40)	3/9 (33.3)	6/19 (31.6)	7/19 (36.8)	$p_4 = 0.405$ $p_5 = 0.599$ $p_6 = 0.764$ $p_7 = 0.926$ $p_8 = 0.439$
ACVA (acute cerebrovascular accident) in past medical history, abs. (%)	0/9 (0)	3/10 (30)	3/9 (33.3)	3/19 (15.8)	6/19 (31.6)	$p_4 = 0.073$ $p_5 = 0.058$ $p_6 = 0.876$ $p_7 = 0.291$ $p_8 = 0.057$
Bronchial asthma, abs. (%)	1/9 (11.1)	1/10 (10)	2/9 (22.2)	2/19 (10.5)	3/19 (15.8)	$p_4 = 0.937$ $p_5 = 0.527$ $p_6 = 0.466$ $p_7 = 0.409$ $p_8 = 0.741$
Lower extremity atherosclerosis, abs. (%)	0/9 (0)	2/10 (20)	4/9 (44.4)	2/19 (10.5)	6/19 (31.6)	$p_4 = 0.156$ $p_5 = 0.023$ $p_6 = 0.252$ $p_7 = 0.041$ $p_8 = 0.057$
Chronic bronchitis, abs. (%)	2/9 (22.2)	2/10 (20)	0/9 (0)	4/19 (21.1)	2/19 (10.5)	$p_4 = 0.906$ $p_5 = 0.134$ $p_6 = 0.156$ $p_7 = 0.137$ $p_8 = 0.409$
Type 2 diabetes mellitus, abs. (%)	0/9 (0)	3/10 (30)	3/9 (33.3)	3/19 (15.8)	6/19 (31.6)	$p_4 = 0.073$ $p_5 = 0.058$ $p_6 = 0.876$ $p_7 = 0.291$ $p_8 = 0.057$
Charlson's comorbidity index (abs.), Me [C25–C75]	9.0 [9.0–9.5]	10.0 [9.0–12.3]	12.0 [10.0–15.5]	10.0 [9.0–11.0]	12.0 [10.0–13.0]	$p_4 = 0.156$ $p_5 = 0.001$ $p_6 = 0.079$ $p_7 = 0.004$ $p_8 = 0.007$
GFR (glomerular filtration rate) (CKD-EPI) (ml/min/1.73 m^2), Me [C25-C75]	47.9 [39.4–59.3]	50.4 [46.8–54.5]	46.8 [37.5–52.4]	48.0 [45.7–56.7]	49.5 [45.4–52.5]	$p_4 = 0.497$ $p_5 = 0.546$ $p_6 = 0.243$ $p_7 = 0.285$ $p_8 = 0.962$
CKD Stage 4 (GFR < 30 ml/min/1.73 m^2), abs. (%)	0/9 (0)	0/10 (0)	1/9 (11.1)	0/19 (0)	1/19 (5.3)	$p_4 = –$ $p_5 = 0.303$ $p_6 = 0.279$ $p_7 = 0.139$ $p_8 = 0.483$
GFR in CKD Stage 4 (ml/min/1.73 m^2), Me [C25–C75]	0	0	26.8	0	26.81	$p_4 = –$ $p_5 = –$ $p_6 = –$ $p_7 = –$ $p_8 = –$
CKD Stage 3B (GFR = 30–44 ml/min/1.73 m^2), abs. (%)	3/9 (33.3)	1/10 (10)	2/9 (22.2)	4/19 (21.1)	3/19 (15.8)	$p_4 = 0.213$ $p_5 = 0.599$ $p_6 = 0.466$ $p_7 = 0.944$ $p_8 = 0.291$

TABLE 11 (*continued*)

GFR in CKD Stage 3B (ml/min/1.73 m^2), Me [C25–C75]	38.6 [34.4–38.6]	41.1	37.4 [36.4–37.4]	39.4 [35.4–40.9]	38.4 [36.4–38.4]	$p_4 = 0.500$ $p_5 = 1.000$ $p_6 = 1.000$ $p_7 = –$ $p_8 = 1.000$
CKD Stage 3A (GFR = 45–59 ml/min/1.73 m^2), abs. (%)	5/9 (55.6)	8/10 (80)	6/9 (66.7)	13/19 (68.4)	14/19 (73.7)	$p_4 = 0.252$ $p_5 = 0.629$ $p_6 = 0.510$ $p_7 = 0.926$ $p_8 = 0.337$
GFR in CKD Stage 3A (ml/min/1.73 m^2), Me [C25–C75]	48.0 [46.8–59.3]	50.4 [47.1–53.1]	51.6 [46.5–53.2]	49.5 [47.4–55.2]	50.9 [46.9–52.8]	$p_4 = 0.833$ $p_5 = 0.792$ $p_6 = 1.000$ $p_7 = 0.533$ $p_8 = 0.754$
CKD Stage 2 (GFR = 60–89 ml/min/1.73 m^2), abs. (%)	1/9 (11.1)	1/10 (10)	0/9 (0)	2/19 (10.5)	1/19 (5.3)	$p_4 = 0.937$ $p_5 = 0.303$ $p_6 = 0.330$ $p_7 = 0.312$ $p_8 = 0.575$
GFR in CKD Stage 2 (ml/min/1.73 m^2), Me [C25–C75]	61.1	65.1	0	63.1 [61.1–68.1]	65.05	$p_4 = 1.000$ $p_5 = –$ $p_6 = –$ $p_7 = 0.898$ $p_8 = 1.000$
CKD Stage 1 (GFR ≥ 90 ml/min/1.73 m^2), abs. (%)	–	–	––	–	–	–
GFR in CKD Stage 1 (ml/min/1.73 m^2), Me [C25–C75]	–	–	–	–	–	–

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_7 – differences between the group of patients taking rivaroxaban without CCBs + patients taking rivaroxaban in combination with DCCBs (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCBs (verapamil); p_8 – differences between the group of patients taking rivaroxaban without CCBs and the group of patients taking rivaroxaban in combination with all CCBs (amlodipine + verapamil); BMI – body mass index; CHA_2DS_2-VASc – scale for assessing the risk of stroke and systemic thromboembolism in patients with atrial fibrillation (Congestive heart failure; Hypertension; Age ≥ 75 years; Diabetes mellitus; prior Stroke or TIA or thromboembolism; Vascular disease; Age 65–74 years; Sex Category); HAS-BLED – scale for assessing the risk of bleeding in atrial fibrillation (Hypertension; Abnormal renal-liver function; Stroke; Bleeding history or predisposition; Labile international normalised ratio; Elderly (65 years); Drugs or alcohol concomitantly); CHD – coronary heart disease; ACVA – acute cerebrovascular accident.

TABLE 12

PECULIARITIES OF DRUG INTERACTION IN PATIENTS CARRYING TT GENOTYPE OF *ABCB1* GENE (rs4148738)

Indicators	TT genotype (n = 28) of the *ABCB1* gene (rs4148738)			*p* value
	Rivaroxaban without CCBs	Rivaroxaban and amlodipine	Rivaroxaban and verapamil	
Number of patients, abs. (%)	9/28 (32.1)	10/28 (35.8)	9/28 (32.1)	–
$C_{min, ss}$ of rivaroxaban (ng/ml), Me [C25–C75]	66.0 [36.4–103.1]	45.1 [20.1–52.8]	82.2 [49.8–120.95]	$p_4 = 0.165$ $p_5 = 0.508$ $p_6 = 0.022$
$C_{min, ss}$/D of rivaroxaban (ng/ml/mg), Me [C25–C75]	3.8 [2.3–6.9]	3.0 [1.2–3.5]	5.1 [3.3–8.1]	$p_4 = 0.221$ $p_5 = 0.402$ $p_6 = 0.022$
Prothrombin time (s), Me [C25–C75]	14.2 [13.6–14.5]	13.1 [12.3–14.6]	17.4 [13.6–18.5]	$p_4 = 0.252$ $p_5 = 0.046$ $p_6 = 0.027$
Data on all clinically significant minor bleeding in response to rivaroxaban by history, abs. (%)	1/9 (11.1)	3/10 (30)	7/9 (77.8)	$p_4 = 0.313$ $p_5 = 0.004$ $p_6 = 0.037$

Note. abs. – absolute number; p_4 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with DCCB (amlodipine); p_5 – differences between the group of patients taking rivaroxaban without CCB and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil); p_6 – differences between the group of patients taking rivaroxaban in combination with DCCB (amlodipine) and the group of patients taking rivaroxaban in combination with non-DCCB (verapamil).

administration of rivaroxaban with verapamil (strong P-gp inhibitor and moderate CYP3A4 inhibitor) resulted in higher values of $C_{min, ss}$ of rivaroxaban compared to patients, not taking CCBs, and compared to patients taking rivaroxaban and amlodipine (potentially possible drug interactions caused by competition of substrates for binding sites on cell membranes). In carriers of the homozygous genotype (TT) of the *ABCB1* gene (rs1045642), the co-administration of rivaroxaban with verapamil was associated with AR in the majority of cases in the form of clinically significant small bleeds (up to 75 %), and their number was statistically significantly higher in comparison with patients not taking CCB, and in comparison with patients taking rivaroxaban and amlodipine.

In patients 80 years and older with non-valvular AF and patients carrying the wild-type genotype (WS) of the *ABCB1* gene (rs4148738), co-administration of rivaroxaban with verapamil resulted in higher $C_{min, ss}$ values of rivaroxaban compared with patients not taking CCB in combination with rivaroxaban. In carriers of heterozygous genotype (CT) of *ABCB1* gene (rs4148738) co-administration of rivaroxaban with CCB did not lead to statistically significant differences between the studied parameters. In carriers of the homozygous genotype (TT) of the *ABCB1* gene (rs4148738), co-administration of rivaroxaban with verapamil resulted in statistically higher AR in the form of clinically significant minor bleeding compared to the group of patients not taking CCB in combination with rivaroxaban (up to 78 %).

We would like to draw the readers' attention to the fact that earlier in our studies we have already revealed that co-administration of verapamil (a strong P-gp inhibitor and moderate CYP3A4 inhibitor) in combination with rivaroxaban resulted in more frequent AR development (in 33 % of patients) [4], and homozygous type (TT) carriers of *ABCB1* gene (rs1045642 and rs4148738) were associated with more frequent AR development (29.3 % and 39.3 %, respectively) [10]. In this study, it was observed that co-administration of verapamil (a strong P-gp inhibitor and moderate CYP3A4 inhibitor) in combination with rivaroxaban in homozygous type (TT) carriers of the *ABCB1* gene (rs1045642 and rs4148738) resulted in AR in 75 and 78 % of cases, respectively. Consequently, it may be advisable to genotype patients to clarify the carriage of polymorphic variants of *ABCB1* gene (rs1045642 and rs4148738) before the administration of verapamil (a strong P-gp inhibitor and moderate CYP3A4 inhibitor) in combination with rivaroxaban and then decide about the further strategy of patient treatment. This study could prevent the development of AR in the form of clinically significant minor bleeding in patients 80 years and older with non-valvular AF.

The fact that more than half of the patients carrying the TT genotype of the *ABCB1* gene (rs1045642 and rs4148738) in the subgroup of patients taking rivaroxaban + verapamil were men, whereas in the subgroups of patients taking rivaroxaban + amlodipine and rivaroxaban without CCB, women predominated (Tables 5, 11), also requires discussion. In our previous study, we demonstrated that there were 10 cases of CSNMB when taking rivaroxaban with verapamil, 8 of which were haematuria [4]. The resolution of the Eurasian Association of Therapists on the algorithm for assessment and modification of risk factors for minor bleeding in patients with AF treated with direct oral anticoagulants (DOACs) states that the most common causes of haematuria in patients taking rivaroxaban or other DOACs are benign prostatic hyperplasia (BPH) and prostatitis [19]. Consequently, it may be assumed that in patients carrying the TT genotype of the *ABCB1* gene (rs1045642 and rs4148738) the sharply pronounced differences in the CPNA incidence in the three selected subgroups may be explained by the heterogeneity of the subgroups by sex composition. The incidence of BPH, however, increases with age, reaching 88 % after the age of 80 years [20]. Considering the fact that all our patients were over 80 years of age, it can be assumed that of the 25 % (32 patients) of men included in the study, 88 % (28 patients) of the men may have BPH, of which 39 % (11 patients) had haematuria, whereas in the population of men over 75 years of age, haematuria occurs in only 13 % of cases [20]. Alternatively, if we consider haematuria as a complication of BPH, then the cause of its occurrence is not only and not so much the disease of the prostate gland itself, but rather the change in bladder function and, as a consequence, dilated varicose veins of the bladder neck, which can also develop as a result of other (not only BPH) causes, such as bladder 'ageing', changes in neurological status, the presence of comorbidities, and therefore occurs with approximately equal frequency in both older men and older women [20]. Nevertheless, to confirm or refute the assumption that the heterogeneity of subgroups by sex composition influenced the sharply pronounced differences in the CPNA incidence among patients carrying the TT genotype of the *ABCB1* gene (rs1045642 and rs4148738) in the three selected subgroups, it is necessary to conduct an additional study that will include a larger number of participants and homogeneous groups by sex composition.

Our data are comparable with the results of a number of foreign studies. Thus, K. Lorenzini et al. [21] reported a case of bleeding in a 79-year-old patient on taking rivaroxaban 20 mg per day (for 3 months). The authors hypothesised that the presence of homozygous TT genotypes for rs2032582 and rs1045642 of the *ABCB1* gene and decreased CYP3A4/5 activity as a result of drug interaction with simvastatin may have contributed to the increased susceptibility to rivaroxaban in the presented patient.

A. Sennesael et al. [22] prospectively analyzed 10 patients admitted to the emergency department for bleeding against the background of rivaroxaban administration. Among the three patients who experienced severe bleeding associated with $C_{min, ss}$ rivaroxaban > 136 ng/ml, two were heterozygous and one was homozygous (TT) for rs1045642 of the *ABCB1* gene. However, no clear association between *ABCB1* genotype and calculated minimum concentrations was observed ($p > 0.050$). At the same time, however, all three patients were also receiving MPs with potential drug

interactions (diltiazem + clarithromycin, or simvastatin, or amiodarone).

In a study by I. Gouin-Thibault et al. [23], it was revealed that *ABCB1* genotype (rs2032582; c.2677G>A/T; p.Ala893Thr/Ser and rs1045642; c.3435C>T; p.Ile1145Ile) is not a significant determinant of individual variability of rivaroxaban pharmacokinetics in healthy volunteers, whereas co-administration of rivaroxaban with a P-gp/CYP3A4 inhibitor (clarithromycin) may increase the risk of overdose, since it increases rivaroxaban's AUC by 94 % ($p < 0.0001$) and its $C_{max, ss}$ by 92 % ($p < 0.0001$): geometric mean ratios were 1.94 [95% confidence interval (95% CI): 1.42–2.63] and 1.92 [95% CI: 1.60–2.28] for AUC and $C_{max, ss}$, respectively, and this effect was not affected by *ABCB1* genotype.

By contrast, P. Pham et al. [24] assessed the risk of bleeding in patients with AF against the background of administering standard doses of DOACs in combination with verapamil or diltiazem. A total of 1764 patients receiving DOACs with verapamil or diltiazem compared with 3105 patients receiving amlodipine and 1793 patients receiving DOACs with verapamil or diltiazem compared with 3224 patients receiving metoprolol were analysed. Results revealed that rivaroxaban and apixaban were not associated with increased bleeding rates in patients receiving verapamil or diltiazem compared to those receiving amlodipine or metoprolol. Among patients receiving dabigatran etexilate, the overall incidence of bleeding was 52 % higher (hazard ratio (HR) – 1.52; 95% CI: 1.05–2.20) when taking verapamil or diltiazem compared with amlodipine and 43 % higher (HR = 1.43; 95% CI: 1.02–2.20) when compared with metoprolol. The incidence of bleeding during the administration of dabigatran with verapamil or diltiazem was generally higher for other types of bleeding (244.9 vs. 158.4 per 1000 person-years; adjusted risk ratio for total gastrointestinal bleeding – 2.16 (95% CI: 1.30–3.60), minor bleeding – 1.56 (95% CI: 1.07–2.27), minor gastrointestinal bleeding – 2.16 (95% CI: 1.29–3.63). Sensitivity analyses revealed consistent results for dabigatran being used with verapamil and diltiazem with hazard rate increase values ranging from 50 % to 100 % and no statistically significant results for apixaban or rivaroxaban. In contrast to this study, in P. Pham et al. patients had no history of chronic kidney disease (CKD) and also 60 % of patients were younger than 65 years with only about 5.5 % older than 80 years; in this study, only 12 % had no history of CKD and all patients were older than 80 years, which could have affected the difference in the results obtained. The results of our study, however, need to be verified in a prospective study with a larger number of participants.

Study limitations

Our study has several limitations. Firstly, the sample of patients in the groups according to the gene polymorphisms under study was small. Second, the baseline characterization of patients was not comparable in a number of parameters. These limitations may have affected the outcome.

CONCLUSION

This study, in which features of drug interaction between rivaroxaban and CCB in patients 80 years and older with non-valvular AF cause-specific polymorphisms of *ABCB1* gene (rs1045642 and rs4148738) were examined, showed statistically significant changes in the pharmacokinetic profile of certain *ABCB1* gene variants (rs1045642 and rs4148738) and, as a consequence, the occurrence of AR in the form of clinically significant minor bleeding. Therefore, to prevent their occurrence in patients 80 years and older with non-valvular AF, genotyping for variants of the above polymorphisms of the *ABCB1* gene (rs1045642 and rs4148738) may be considered before prescribing verapamil (a strong P-gp inhibitor and a moderate CYP3A4 inhibitor).

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Dmitry A. Sychev – Dr. Sc. (Med.), Professor, Professor of the RAS, Member of the RAS, Honored Scientist of the Russian Federation, Head of the Academician B.E. Votchal Department of Clinical Pharmacology and General Medicine, Rector, Russian Medical Academy of Continuous Professional Education, e-mail: dimasychev@mail.ru, https://orcid.org/0000-0002-4496-3680

Karin B. Mirzaev – Dr. Sc. (Med.), Vice Rector for Science and Innovation, Russian Medical Academy of Continuous Professional Education, e-mail: karin05doc@yandex.ru, https://orcid.org/0000-0002-9307-4994

Marina S. Cherniaeva – Cand. Sc. (Med.), Associate Professor at the Department of Internal Diseases and Preventive Medicine, Central State Medical Academy of the Administrative Directorate of the President of the Russian Federation; Head of the Department of Geriatrics, Geriatrician, General Physician, War Veterans Hospital No. 2 of the Department of Health Services of Moscow, e-mail: doctor@cherniaeva.ru, https://orcid.org/0000-0003-3091-7904

Nataliia V. Shakhgildian – Resident Physician, Lomonosov Moscow State University, e-mail: shakhgildian@gmail.com, https://orcid.org/0000-0002-7367-6461

Sherzod P. Abdullaev – Cand. Sc. (Biol.), Head of the Department of Predictive and Prognostic Biomarkers, Research Institute for Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: abdullaevsp@gmail.com, https://orcid.org/0000-0001-9001-1499

Natalia P. Denisenko – Cand. Sc. (Med.), Deputy Director, Research Institute for Molecular and Personalized Medicine, Associate Professor at the Academician B.E. Votchal Department of Clinical Pharmacology and General Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: natalypilipenko3990@gmail.com, https://orcid.org/0000-0003-3278-5941
Zhannet A. Sozaeva – Junior Research Officer at the Department of Predictive and Prognostic Biomarkers, Research Institute for Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: zhannet.sozaeva@yandex.ru, https://orcid.org/0000-0001-5166-7903
Anastasia A. Kachanova – Junior Research Officer at the Department of Predictive and Prognostic Biomarkers, Research Institute for Molecular and Personalized Medicine, Russian Medical Academy of Continuous Professional Education, e-mail: aakachanova@yandex.ru, https://orcid.org/0000-0003-3194-4410
Vera R. Shastina – Cand. Sc. (Med.), Associate Professor at the Department of N.A. Semashko Department for Public Health and Healthcare, I.M. Sechenov First Moscow State Medical University (Sechenov University); Chief Physician, War Veterans Hospital No. 2 of the Department of Health Services of Moscow, e-mail: vershast@mail.ru, https://orcid.org/0000-0002-2933-7876
Svetlana V. Gorbatenkova – Cand. Sc. (Med.), Deputy Chief Physician for Medicine, War Veterans Hospital No. 2 of the Department of Health Services of Moscow, e-mail: gorbatenkova_sv@mail.ru, https://orcid.org/0000-0003-0804-4182

PRODUCTION OF ANGIOGENESIS MEDIATORS AND THE STRUCTURE OF THE VASCULAR WALL IN THE HEART IN ISCHEMIC CARDIOMYOPATHY

Chumakova S.P. [1],
Urazova O.I. [1],
Shipulin V.M. [1],
Sukhodolo I.V. [2],
Stelmashenko A.I. [1],
Denisenko O.A. [1],
Andreev S.L. [2],
Demin M.S. [1],
Churina E.G. [1, 3]

[1] Siberian State Medical University (Moskovsky Trakt 2, Tomsk 634050, Russian Federation)
[2] Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences (Kievskaya str. 111A, Tomsk 634012, Russian Federation)
[3] National Research Tomsk State University (Lenina Ave. 36, Tomsk 634050, Russian Federation)

Corresponding author:
Olga A. Denisenko,
e-mail: olga-muraveinik@yandex.ru

ABSTRACT

Background. *In the pathogenesis of ischemic cardiomyopathy (ICMP), angiopoiesis remains unexplored.*
The aim of the study. *To describe the vasculature of the heart and the imbalance of angiogenesis mediators in the coronary circulation in association with the number of endothelial progenitor cells (EPC) and desquamated endothelial cells (DEC) in the blood of patients with coronary heart disease (CHD), suffering and not suffering from ICMP.*
Materials and methods. *Fifty-two patients with CHD (30 patients with ICMP, 22 patients without ICMP), 15 healthy donors were examined. The content of EPC ($CD14^+CD34^+VEGFR2^+$) in the blood from the cubital vein and DEC ($CD45^-CD146^+$) in the blood from the coronary sinus and the cubital vein was determined by flow cytometry. The concentrations of VEGF-A (vascular endothelial growth factor A), PDGF (platelet-derived growth factor), and SDF-1 (stromal cell-derived factor 1) in blood plasma were recorded using immunofluorescence assay; the angiopoietin-2, MMP-9 (matrix metallopeptidase 9) were recorded using enzyme immunoassay. In myocardial biopsies the specific area of vessels and the expression of αSMA (smooth muscle alpha-actin) were determined by morphometric and immunohistochemical methods.*
Results. *In the peripheral blood of patients with CHD, regardless of the presence of ICMP, the DEC content exceeded the physiological level, and the VEGF-A, PDGF, angiopoietin-2, and MMP-9 corresponded to the norm. In CHD patients without cardiomyopathy, there was an excess of SDF-1 and EPC in the blood from the cubital vein, and in ICMP, their physiological significance was noted. In the coronary blood flow in patients with CHD without cardiomyopathy, an increase in the concentration of PDGF was found, which was not determined in patients with ICMP, who had an increased content of DEC, angiopoietin-2 and MMP-9. The specific area of the vessels in the patients of the two groups was comparable; the expression of αSMA in ICMP was 6.2 times lower than in patients with CHD without cardiomyopathy.*
Conclusion. *The development of ICMP is accompanied by impaired maturation of vessels in the myocardium, associated with the absence of a compensatory reaction of activation of cellular and humoral factors of angiogenesis.*

Key words: *angiogenesis, growth factors, endothelial progenitor cells, myocardium, coronary heart disease*

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ПРОДУКЦИЯ МЕДИАТОРОВ АНГИОГЕНЕЗА И СТРУКТУРА СОСУДИСТОЙ СТЕНКИ В СЕРДЦЕ ПРИ ИШЕМИЧЕСКОЙ КАРДИОМИОПАТИИ

Чумакова С.П. [1],
Уразова О.И. [1],
Шипулин В.М. [2],
Суходоло И.В. [1],
Стельмашенко А.И. [1],
Денисенко О.А. [1],
Андреев С.Л. [2],
Дёмин М.С. [1],
Чурина Е.Г. [1, 3]

[1] ФГБОУ ВО «Сибирский государственный медицинский университет» Минздрава России (634050, г. Томск, Московский тракт, 2, Россия)
[2] Научно-исследовательский институт кардиологии, ФГБУ «Томский национальный исследовательский медицинский центр Российской академии наук» (634012, г. Томск, ул. Киевская, 111а, Россия)
[3] ФГАОУ ВО «Национальный исследовательский Томский государственный университет» (634050, г. Томск, просп. Ленина, 36, Россия)

Автор, ответственный за переписку:
Денисенко Ольга Анатольевна,
e-mail: olga-muraveinik@yandex.ru

РЕЗЮМЕ

Актуальность. *При ишемической кардиомиопатии (ИКМП) ангиогенез остаётся неизученным.*
Цель исследования. *Охарактеризовать сосудистую сеть сердца и дисбаланс медиаторов ангиогенеза в коронарном кровотоке в ассоциации с численностью эндотелиальных прогениторных клеток (ЭПК) и десквамированных эндотелиальных клеток (ДЭК) в крови у больных ишемической болезнью сердца (ИБС), страдающих и не страдающих ишемической кардиомиопатией.*
Методы. *Обследованы 52 больных ИБС (30 пациентов с ИКМП, 22 пациента без ИКМП) и 15 здоровых доноров. В крови из кубитальной вены определяли содержание ЭПК ($CD14^+CD34^+VEGFR2^+$), из коронарного синуса и кубитальной вены – ДЭК ($CD45^-CD146^+$) методом проточной цитофлуориметрии. В плазме крови регистрировали концентрацию фактора роста эндотелия сосудов A (VEGF-A, vascular endothelial growth factor A), фактора роста тромбоцитов (PDGF, platelet-derived growth factor), стромального клеточного фактора 1 (SDF-1, stromal cell-derived factor 1) с помощью иммунофлуоресцентного анализа; ангиопоэтина-2, матриксной металлопротеиназы 9 (MMP-9, matrix metallopeptidase 9) – методом иммуноферментного анализа. В биоптатах миокарда определяли удельную площадь сосудов и экспрессию αSMA (smooth muscle alpha-actin) морфометрическим и иммуногистохимическим методами.*
Результаты. *В периферической крови у больных ИБС вне зависимости от наличия ИКМП содержание ДЭК превышало физиологический уровень, а содержание VEGF-A, PDGF, ангиопоэтина-2 и MMP-9 соответствовало норме. У больных ИБС без кардиомиопатии в крови из кубитальной вены отмечался избыток SDF-1 и ЭПК, а при ИКМП – их физиологическое значение. В коронарном кровотоке у больных ИБС без кардиомиопатии установлено повышение концентрации PDGF, чего не определялось у пациентов с ИКМП, у которых было увеличено содержание ДЭК, ангиопоэтина-2 и MMP-9. Удельная площадь сосудов у больных двух групп была сопоставимой, экспрессия αSMA при ИКМП была в 6,2 раза ниже, чем у больных ИБС без кардиомиопатии.*
Заключение. *Развитие ИКМП сопровождается нарушением созревания сосудов в миокарде, связанным с отсутствием компенсаторной реакции активации клеточных и гуморальных факторов ангиогенеза.*

Ключевые слова: *ангиогенез, факторы роста, эндотелиальные прогениторные клетки, миокард, ишемическая болезнь сердца*

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INTRODUCTION

Ischemic cardiomyopathy (ICMP) is a severe disease that has no specific pharmacotherapy to date and is characterised by disease progression in some patients even after surgical correction of the coronary bed and left ventricular cavity [1, 2]. This demonstrates the insufficiently studied pathogenesis of ICMP, in which the role of chronic inflammation, cardiomyocyte apoptosis, disorders of Ca^{2+} homeostasis and myocardial contractile function, synthesis of different types of collagens, and microvascular dysfunction have been actively discussed to date [2–4]. Therewith, the interest of scientists is focused on the vasomotor form of endothelial dysfunction [5, 6]. Angiogenic form of endothelial dysfunction in ICMP, including impaired angiogenesis, balance of reparative and destructive processes in vessels [7], however, has not been studied.

Both forms of chronic coronary heart disease (CHD) are accompanied by damage to the vascular intima, since the morphological substrate of CHD, either complicated or uncomplicated by ICMP, is atherosclerosis of the coronary arteries. On the one hand, plaque macrophages support chronic inflammation, prolong vascular alteration and endothelial desquamation with the help of matrix metalloproteinases (MMP) [1, 8, 9], but they also contribute to atheroma vascularisation, which increases the risk of plaque haemorrhage with its subsequent destabilisation [5, 10]. On the other hand, induction of angiogenesis is necessary for formation of collateral blood flow and repair of damaged vessels, which has protective and adaptive value in CHD and ICMP. Angiogenesis is performed by endothelial progenitor cells (EPC), most of which have a monocytic immunophenotype and reparative potential in relation to endothelium as a result of paracrine secretion of angiogenesis factors [11].

In this regard, studying the output of such mediators of angiogenesis as vascular endothelial growth factor (VEGF) A, platelet-derived growth factor (PDGF), stromal cell-derived factor (SDF) 1, angiopoietin (Ang) 2 and MMP-9 in the heart [11, 12] can establish the mechanisms of angiogenesis and angiopoietic endothelial dysfunction in CHD both complicated and uncomplicated by ICMP. At the same time, comparison of the number of EPCs of monocytic immunophenotype and desquamated endothelial cells (DEC) in blood, as well as determination of the specific volume of vessels and expression of alpha-smooth muscle actin (αSMA), which is synthesized by vascular smooth muscle cells [13], will enable to determine the correlation of angiogenesis factors with the degree of coronary endothelial damage in ICMP relative to CHD without cardiomyopathy.

THE AIM OF THE STUDY

To reveal features of vascular network formation in the heart and imbalance of angiogenesis mediators in coronary blood flow in association with the number of endothelial progenitor and desquamated cells in blood from patients affected by coronary heart disease with and without ischemic cardiomyopathy.

METHODS

A single-stage controlled (case-control) single-center observational study was conducted from February 2020 to May 2022. The study included 52 CHD patients with tension angina II–IV functional class and circulatory insufficiency, mainly II–III functional class according to NYHA (New York Heart Association), who had a history of myocardial infarction and were hospitalised at the Research Institute of Cardiology of Tomsk National Research Medical Centre of the Russian Academy of Sciences for the purpose of coronary bypass surgery. Patients with CHD were categorised into two groups: 30 patients with ICMP (27 men and 3 women; mean age – 61.0 [56.0; 64.0] years) and 22 patients without cardiomyopathy (18 men and 4 women; mean age – 64.0 [59.5; 67.0] years). According to the criteria of G.M. Felker et al. (2002), the signs of ICMP were low left ventricular ejection fraction (less than 40 %), haemodynamically significant stenosis of two or more epicardial vessels or the trunk of the left descending artery [14]. CHD patients without cardiomyopathy had similar coronary vessel changes but had preserved left ventricular ejection fraction (more than 40 %). The control group consisted of 15 practically healthy donors (13 men and 2 women; mean age – 57.63 ± 8.12 years) without any cardiovascular diseases and relevant complaints.

CHD patients both with and without ICMP were comparable in terms of age, sex, body mass index, duration of CHD, functional class of angina and circulatory insufficiency, as well as frequency of prescription of statins. However, they were statistically significantly different in terms of left ventricular parameters: ICMP patients compared to CHD patients without cardiomyopathy had higher myocardial mass (233.0 [221.7; 266.2] g vs. 184.0 [140.5; 214.5] g; $p < 0.001$) but lower ejection fraction (30.00 [22.00; 36.00] % vs. 59.50 [50.25; 67.00] %; $p < 0.001$), as a decrease in the latter less than 40 % was a criterion for ICMP diagnosis and patient grouping. The pattern of comorbidity in the patient cohorts was also comparable, except for a higher incidence of type 2 diabetes mellitus in CHD patients without ICMP (31.82 % vs. 6.67 %; $p = 0.046$) and chronic cerebral circulatory disorders in patients with ICMP (90.0 % vs. 59.1 %; $p = 0.023$).

All CHD patients underwent coronary artery bypass surgery with similar anaesthetic management (diazepam, ketamine, fentanyl, promedol, pipecuronium). At the preoperative stage, patients of both groups were treated according to the generally accepted principles of CHD therapy (prolonged-acting nitrates and on demand – calcium channel blockers, β1-adrenoblockers, statins, antiaggregants). Therapy was similar in the CHD patient groups, except for more frequent use of calcium channel blockers in CHD patients without cardiomyopathy, compared with ICMP patients (63.6 % vs. 0 %; $p < 0.001$). More frequent prescription of anticoagulants in CHD patients without cardiomyopathy may

be associated with a greater intensity of atherogenesis and involvement of lower limb vessels than in ICMP.

The exclusion criteria of patients from the study were as follows: age older than 70 years; presence of allergic disease in the exacerbation stage, autoimmune diseases, anaemia, tumour process, syphilis, HIV infection, viral hepatitis; presence of acute infectious diseases less than 3 weeks before surgery; prescription of erythropoietin or immunosuppressive therapy; patient's refusal to participate in the study.

The studies were conducted in accordance with the ethical principles outlined in the World Medical Association Declaration of Helsinki (1975) and with the permission of the local Ethical Committee of the Siberian State Medical University of the Ministry of Health of Russia (Protocol No. 7981 dated December 16, 2019). Informed consent for participation in the study was obtained from all individuals examined.

The study material included blood samples from the cubital vein (peripheral blood) and blood from the coronary sinus (sinus blood) stabilized with heparin (25 IU/ml), as well as biopsy specimens of the auricle of the right atrium. Peripheral blood was collected in a volume of 5 ml from the cubital vein in the morning on an empty stomach in both healthy donors and CHD patients of both study groups on the day of surgery immediately before induction into anaesthesia. Peripheral blood was used for immunophenotyping of EPCs and DECs, its plasma was used to estimate the concentration of the studied mediators. Blood from the coronary sinus in the volume of 5 ml was obtained only in CHD patients: intraoperatively, by transmyocardial puncture after surgical access to the heart, but before connection of the artificial circulation device and the main stage of the surgery. DEC content was determined in blood from the coronary sinus, blood plasma from the coronary sinus was used to study the concentration of the studied mediators. Myocardial biopsies of the right atrial auricle in volume not more than 10 mm^3 were obtained intraoperatively at the stage of its cannulation for connection of the artificial circulation device, but before the commencement of extracorporeal perfusion. Myocardial biopsy specimens were used to determine specific vessel area by morphometric method and α-SMA expression by immunohistochemical method.

DEC absolute amount and EPC relative content in blood were determined by flow cytofluorimetry in venous blood obtained from the cubital vein in healthy donors and in CHD patients of both groups (peripheral blood). In patients, DEC content was also assessed in blood from the coronary sinus. Whole blood was lysed by adding FACS Lysing solution (BD Biosciences, USA), then cells were washed three times with 20-fold volume of Cell-WASH-solution BD buffer (Becton Dickinson, USA). Mouse Anti-Human CD14-FITC, CD34-PE, VEGFR2(KDR; CD309)-Alexa Fluor 647, CD45-FITC and CD146-Alexa Fluor 647 monoclonal antibodies were used to detect EPCs with $CD14^+CD34^+VEGFR2^+$ immunophenotype and DECs with $CD45^-CD146^+$ immunophenotype, according to the manufacturer's instructions (BD Biosciences, USA). Fluorescence intensity measurements were performed using an Accuri C6 flow cytometer (BD Biosciences, USA), and the data were analyzed using BD Cell Quest for Mac OS® X software application (BD Biosciences, USA). DEC fraction among all blood cells analysed was correlated with the total number of leukocytes expressing $CD45^+$ (CD45 – total leukocyte antigen), expressed in $\times 10^5$/l. The total number of leukocytes in blood was assessed by flow cytofluorimetry using a XS-1000i haematological analyzer (Sysmix Corporation, Japan).

Peripheral blood plasma from CHD patients of both study groups and healthy donors, as well as blood plasma from the coronary sinus of CHD patients of both groups was aliquoted and stored at –80 °C for no more than 12 months. The concentration of VEGF-A, PDGF, SDF-1 was measured using a commercial multiplex assay test system 'Magnetic Luminex Assay Kit for VEGFA, VEGFB, PDGF, SDF1, SCF, FGF1, GM-CSF, MSR1' (Cloud-Clone Corp., USA) and an automated analyzer Bio-Plex Protein Assay System (Bio-Rad, USA). The concentration of Ang-2 and MMP-9 proteinase in plasma was measured by enzyme-linked immunosorbent assay using commercial kits 'RayBio Human ANGPT2 ELISA Kit' (RayBiotech, USA) and 'Human MMP9 ELISA' (ThermoFisher Scientific, USA) according to the manufacturers' instructions.

The myocardial samples obtained were fixed in 10 % neutral buffered formalin, paraffinized and histological sections 4–5 μm thick were made using an automatic rotary microtome HM 355 S (Thermo Scientific, USA). Sections were stained with hematoxylin and eosin [15], enclosed in BioMount mounting medium (BioOptica, Italy). Immunohistochemical staining was performed on 4 μm thick paraffin sections for which deparaffinization, antigen demasking, and blocking of non-specific binding with 3 % bovine serum albumin in phosphate-buffered saline (PBS) were performed. Following this, slices were incubated with primary antibodies to αSMA (Spring BioScience, USA) for 60 min in a humid chamber followed by 3-fold washing in PBS, then incubated with secondary HRP-labelled antibodies for 45 min followed by 3-fold washing in PBS. In the last step, DAB-chromagen substrate (HRP-DAB (horseradish peroxidase – diaminobenzidine) imaging system; DAKO, USA) was added and stained with haematoxylin. All sections were enclosed in BioMount mounting medium (BioOptica, Italy). The preparations were studied in transmitted light using an Axioskop 40 microscope (Carl Zeiss, Germany); images were digitized using a Canon G 10 camera (Canon, Japan). Tissue markers were counted at ×400 magnification in 10 randomly selected fields of view corresponding to 1 mm^2 of tissue [16]. Specific vessel area and αSMA expression as percentage of the studied tissue area were estimated using the AxioVision graphic image processing program (Carl Zeiss, ImageJ).

Statistical analysis of the data was performed using Statistica 10.0 program (StatSoft Inc., USA). In statistical description of the results, median, 25th and 75th percentiles were calculated for quantitative traits; for qualitative traits, sample fraction was estimated. In order to comparatively analyze sample data, Mann – Whitney (for independent samples) and Wilcoxon (for dependent samples)

criteria were applied, using Benjamini – Hochberg correction for multiple comparisons. Chi-square test with Yeats' correction for continuity was applied to compare the frequencies of occurrence of the trait in the groups. The results of statistical analysis were considered statistically significant at $p < 0.05$.

RESULTS

The DEC content in peripheral blood of CHD patients, whether ICMP was present or not, was higher than that of healthy donors and did not differ between patient groups in both blood from the cubital vein (Table 1) and blood from the coronary sinus (Table 2). At the same time, EPC abundance in peripheral blood was elevated in CHD patients without cardiomyopathy (Table 1). In patients with ICMP, on the contrary, this parameter of total blood flow varied within physiological values (Table 1), while DEC abundance in sinus blood was 2.5 times higher than in peripheral blood, which was not observed in CHD patients without cardiomyopathy (Tables 1, 2).

The content of VEGF-A and PDGF growth factors in peripheral blood of CHD patients corresponded to the values in healthy donors irrespective of ICMP presence and did not differ between the patient groups (Table 1), but the coronary blood flow analysis revealed significant differences (Table 2). For instance, in CHD patients without cardiomyopathy, PDGF levels were higher in sinus blood than in peripheral blood (Tables 1, 2). Meanwhile, VEGF-A content in blood from the coronary sinus prevailed over its level in blood from the cubital

TABLE 1

CONTENT OF ENDOTHELIAL PROGENITOR AND DESQUAMATED CELLS AS WELL AS MEDIATORS OF ANGIOGENESIS IN BLOOD FROM THE CUBITAL VEIN IN CHD PATIENTS AFFECTED AND NOT AFFECTED BY ICMP, Me [Q1; Q3]

Indicators	Group of examined individuals		
	CHD without ICMP	CHD with ICMP	Healthy donors
EPC content $VEGFR2^+CD34^+CD14^+$, %	0.74 [0.46; 1.23] $\boldsymbol{p_k < 0.001}$	0.31 [0.15; 0.64] $p_k = 0.260$ $\boldsymbol{p = 0.038}$	0.19 [0.13; 0.32]
DECs number $CD45^-CD146^+$, $\times 10^5$/l	7.25 [6.80; 7.47] $\boldsymbol{p_k = 0.038}$	7.26 [5.43; 17.94] $\boldsymbol{p_k = 0.037}$ $p = 0.597$	5.12 [3.73; 5.84]
VEGF-A, pg/ml	4.50 [3.00; 8.00] $p_k = 0.314$	6.00 [3.00; 9.50] $p_k = 0.216$ $p = 0.502$	3.80 [1.00; 6.50]
SDF-1, pg/ml	60.00 [50.00; 80.00] $\boldsymbol{p_k = 0.042}$	49.00 [37.00; 56.00] $p_k = 0.174$ $p = 0.115$	30.00 [5.00; 45.00]
PDGF, pg/ml	3.10 [2.10; 7.05] $p_k = 1.000$	4.85 [1.20; 9.10] $p_k = 1.000$ $p = 0.870$	2.68 [1.65; 7.10]
Angiopoetin-2, pg/ml	445.0 [137.5; 552.5] $p_k = 1.000$	540.0 [403.0; 670.0] $p_k = 0.612$ $p = \mathbf{0}.884$	388.0 [317.0; 460.0]
MMP-9, pg/ml	11.95 [7.00; 13.40] $p_k = 0.460$	13.65 [6.50; 19.60] $p_k = 0.848$ $p = 0.588$	13.20 [9.60; 19.00]

Note. p_k – level of statistical significance of the differences in indicators compared with the content of cytokines/cells in healthy donors; p – level of statistical significance of the differences in indicators compared with the content of cytokines/cells in CHD patients without cardiomyopathy; statistically significant differences are marked in bold.

TABLE 2

DESQUAMATED ENDOTHELIAL CELLS AND MEDIATORS OF ANGIOGENESIS IN BLOOD FROM THE CORONARY SINUS IN ASSOCIATION WITH SPECIFIC VESSEL AREA CHARACTERIZATION AND ASMA EXPRESSION IN MYOCARDIUM FROM CHD PATIENTS BOTH AFFECTED AND NOT AFFECTED BY ICMP, Me [Q1; Q3]

Indicators	Group of examined individuals	
	CHD without ICMP	CHD with ICMP
DECs number $CD45^{-}CD146^{+}$, $\times 10^{5}$/l	10.17 [6.80; 18.83] $p_1 = 0.128$	17.98 [10.27; 22.97] **$p_1 = 0.036$** $p = 0.156$
VEGF-A, pg/ml	7.80 [3.25; 9.75] **$p_1 = 0.041$**	6.89 [3.25; 15.60] **$p_1 = 0.007$** $p = 0.918$
SDF-1, pg/ml	40.30 [26.00; 62.00] **$p_1 = 0.086$**	46.80 [32.50; 64.00] $p_1 = 0.286$ $p = 0.623$
PDGF, pg/ml	7.60 [3.70; 9.94] **$p_1 = 0.036$**	7.86 [2.92; 8.77] $p_1 = 0.674$ $p = 0.736$
Angiopoietin-2, pg/ml	767.0 [494.0; 988.0] $p_1 = 0.128$	1111.5 [845.0; 1235.0] **$p_1 < 0.001$** **$p = 0.002$**
MMP-9, pg/ml	5.92 [5.07; 17.42] $p_1 = 0.972$	16.64 [6.63; 29.12] $p_1 = 0.649$ **$p = 0.038$**
Vessel specific area, %	5.70 [5.60; 6.70]	6.60 [4.60; 8.90] $p = 0.815$
αSMA expression, %	8.10 [7.60; 11.30]	1.30 [0.60; 2.80] **$p = 0.007$**

Note. p_1 – level of statistical significance of the differences in indicators compared with the content of cytokines/cells in peripheral blood; p – level of statistical significance of the differences in indicators compared with the content of cytokines/cells in CHD patients without cardiomyopathy; statistically significant differences are marked in bold.

vein in CHD patients of both groups without differences between patient cohorts (Tables 1, 2). SDF-1 concentrations in peripheral blood exceeded the norm only in CHD patients without cardiomyopathy (Table 1); however, regardless of the ICMP occurrence, the level of this mediator corresponded to that in sinus blood, and no differences between the patient groups were revealed in both blood samples (Tables 1, 2).

The content of Ang-2 and MMP-9 in peripheral blood of CHD patients affected and not affected by ICMP was registered at the level of parameters of healthy donors and did not reveal any differences between patient groups (Table 1). Meanwhile, the concentration of both mediators in blood from the coronary sinus was higher in patients with ICMP than in CHD patients without cardiomyopathy (Table 2). Furthermore, the concentration of Ang-2 in sinus blood prevailed over that in peripheral blood only in patients with ICMP, and the content of MMP-9 corresponded to its content in peripheral blood independently from the ICMP occurrence (Tables 1, 2).

The study of histological preparations of myocardium revealed that the vessel specific area in CHD patients of both study groups was determined at a comparable level, but αSMA expression in patients with ICMP was 6.2 times lower than in CHD patients without cardiomyopathy (Table 2).

DISCUSSION

The obtained data demonstrates significant differences in the mediator profile of blood from the coronary sinus in CHD patients affected and not affected by ICMP, which does not correspond to the nature of the imbalance of angiogenesis factors in peripheral blood (Tables 1, 2), indicating the involvement of different mechanisms of angiogenesis regulation in the affected heart and at the systemic level. Specifically, in patients with ICMP, DEC content in blood from the coronary sinus was higher than in peripheral blood (Tables 1, 2), and EPC level in blood from the cubital vein remained normal (Table 2). In contrast, in CHD patients without cardiomyopathy, DEC abundance in blood samples was comparable (Tables 1, 2) with high levels of EPCs in the systemic bloodstream (Table 2). This indicates an increased attraction of EPCs with reparative potential from bone marrow into blood in CHD patients without cardiomyopathy, which is a compensatory reaction during atherogenesis and, obviously, provides reparative angiogenesis adequate to endothelial destruction in the heart. In ICMP patients this compensatory reaction, apparently, is not realized: physiological level of EPC in blood is insufficient for coronary vessels repair in conditions of atherosclerosis, therefore angiogenesis is not effective, and endothelial destruction prevails, which proves the presence of angiopoietic endothelial dysfunction in ICMP. Significantly, no increased destruction of coronary endothelium was revealed by measurement of DEC content in blood from the cubital vein in ICMP (Table 1).

HIF-1 (hypoxia-inducible factor 1) is a central regulator of angiogenesis as it enhances gene transcription of several pro-angiogenic proteins (SDF-1, VEGF, PDGFB, Ang-1, Ang-2) and their receptors [17], thereby preventing myocardial ischaemic damage [18]. Insufficient coronary vascular repair in ICMP may be associated with an imbalance of angiogenesis mediators that ensure EPC mobilization from the bone marrow, their homing and proliferation/differentiation/secretory activity in coronary vessels. Among the studied mediators of angiogenesis in the peripheral blood of CHD patients without cardiomyopathy manifesting EPC excess, only SDF-1 concentration was elevated, while in patients with ICMP both indices (EPC and SDF-1 content) were in compliance with the norm (Table 1). Accumulation of SDF-1 in plasma stimulates mobilization of $CXCR4^+$ cells from the bone marrow, including haematopoietic stem cells and EPCs, which express CXCR4 as a receptor for SDF-1. The interaction between SDF-1 and CXCR4 also stimulates the recruitment and retention of stem cells in ischaemic areas [12, 19].

The content of another angiogenesis activator VEGF-A in blood from the coronary sinus exceeded that in peripheral blood among CHD patients of both groups (Tables 1, 2), apparently reflecting the induction of angiogenesis under ischaemic conditions. VEGF-A binds to VEGFR1 and VEGFR2, stimulating proliferation and differentiation of EPCs into endothelial cells, formation of tubular structures and increased permeability of the vascular wall, and inhibits cardiomyocyte apoptosis [12, 20–22]. Since hypoxia increases the expression of VEGFR1 [21], which is a trap receptor for VEGF-A and can inhibit angiogenesis [22] and activate MMP-9 secretion from vascular myocytes [23], the interaction of VEGF-A with VEGFR1 may be enhanced in ICMP patients considering widespread myocardial ischaemia, explaining the lack of increase in its blood concentration. In addition to the proangiogenic VEGF-Axxxa family, there is also a family of VEGF-Axxxb isoforms that inhibit angiogenesis [23]. The synthesis of the latter increases under the action of transforming growth factor (TGF) β [23], which is actively secreted in the myocardium of ICMP patients [24]. Additionally, VEGF-A has proatherogenic properties (accumulates triacylglycerols, inhibits lipoprotein lipase), in contrast to VEGF-B, which is characterised by hypolipidemic effects [22].

Along with that, the increase of PDGF concentration in sinus blood relative to peripheral blood in CHD patients without cardiomyopathy (Tables 1, 2) indicates stabilization of newly formed vessels in the heart with VEGF-A participation, which probably does not occur in ICMP patients. PDGF is known to promote not only differentiation, mobilization of EPCs from bone marrow and their migration [25], but also vascular maturation since, unlike VEGF, it attracts pericytes [26], vascular smooth muscle cells and stimulates endothelial-mesenchymal transition [27]. It is being activated in the vascular wall and represents the process of loss of EPC endothelial phenotype and their transdifferentiation into smooth muscle cells, but in dilated cardiomyopathy it is also accompanied by the transition of EPCs into myofibroblasts [28]. PDGF addition to smooth muscle cell culture *in vitro* increases their survival through activation of a signalling pathway involving Notch3, and stimulation of Notch1 signalling maintains their contractile phenotype [13]. This explains the higher expression of αSMA in the myocardium of CHD patients without cardiomyopathy compared with ICMP patients (Table 2). The αSMA protein is synthesized by vascular smooth muscle cells, which are the most numerous in the vascular wall, providing sustenance of vascular tone [13, 28]. Considering that the specific vascular area in CHD patients both without and with ICMP was comparable, and αSMA expression was lower in patients with ICMP (Table 2), therefore, it can be concluded that vascular volume is not altered in ICMP, but the structure of the vascular wall is obviously disturbed. Specifically, in ICMP, newly formed vessels are immature, and existing vessels are likely to lose tone, exacerbating ischaemia and causing myocardial contractile dysfunction and progression of heart failure.

Ang-2 is a negative regulator of angiogenesis since it blocks the binding of proangiogenic Ang-1 to their common receptor Tie-2, destabilises early vessels, and increases their permeability [29]. Ang-2 in conditions of VEGF-A excess, however, can be a Tie-2 agonist and activate angiogenesis, and in the absence of VEGF-A excess, Ang-2 accumulation is associated with vascular regression [23]. Therefore, increased

Ang-2 concentration in sinus blood in patients with ICMP compared with CHD patients without cardiomyopathy, while the level of VEGF-A in coronary blood flow was comparable between them (Table 2), can be considered as a sign of impaired angiogenesis in ICMP. Ang-2 and MMP-9 are considered as markers of cardiovascular disease, atherosclerosis and endothelial dysfunction [6]. MMP-9 degrades extracellular matrix components, including fibronectin [24, 30], which is part of the basolateral membrane of blood vessels [31]. This can either promote angiogenesis or vascular damage [6, 30]. Considering that in ICMP patients the content of MMP-9 and DEC in sinus blood was higher than in peripheral blood, while in CHD patients without cardiomyopathy it was the same (Tables 1, 2), the hypersecretion of MMP-9 in myocardium probably indicates its angiodestructive effect.

The results of the study may be limited by the clinical status of patients, provided that the data obtained are valid for CHD patients with haemodynamically significant multivessel lesions of the main coronary arteries. Consequently, these patterns may not yet be evident in patients in the early stages of ICMP formation, which requires further studies. The results were obtained for individuals of Caucasoid origin living predominantly in the Siberian Federal District.

CONCLUSION

To date, studies of ICMP mechanisms consider the imbalance of different types of collagen, cardiomyocyte apoptosis, impaired Ca^{2+} homeostasis and myocardial contractile function, vasomotor dysfunction of microvessels as its pathogenetic factors. However, the mechanisms of angiogenesis in ICMP patients have not been studied before. The present study has revealed that in CHD, complicated and uncomplicated by ICMP, two different variants of its pathogenesis are realized: with and without impaired angiogenesis. The progression of CHD without cardiomyopathy is accompanied by a compensatory increase in the mobilization of EPCs from the bone marrow in response to atherogenesis by excess SDF-1 in the blood. EPCs are actively recruited to the heart by VEGF-A and PDGF. Mature vessels containing sufficient smooth muscle cells (expressing αSMA) are formed in the myocardium as a result of PDGF secretion; therefore, activation of angiogenesis limits the progression of ischaemia and endothelial desquamation remains moderate. ICMP formation is associated with the absence of increased EPC mobilization, which are attracted to the myocardium by the action of VEGF-A alone, where, without the involvement of PDGF, immature vessels are formed that are easily degraded by Ang-2 and MMP-9. Such angiogenesis is obviously inadequate to the degree of vascular damage and forms a vicious circle of myocardial ischaemia in ICMP. The obtained knowledge about the mechanisms of dysregulation of angiogenesis in ICMP defines targets for its angiogenic therapy, the development of which will enable to slow down the progression of this severe disease.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Svetlana P. Chumakova – Dr. Sc. (Med.), Docent, Professor at the Department of Pathophysiology, Research Officer at the Central Research Laboratory, Siberian State Medical University, e-mail: chumakova_s@mail.ru, https://orcid.org/0000-0003-3468-6154

Olga I. Urazova – Dr. Sc. (Med.), Professor, Corresponding Member of the RAS, Head of the Department of Pathophysiology, Researcher at the Central Research Laboratory, Siberian State Medical University, e-mail: urazova72@yandex.ru, https://orcid.org/0000-0002-9457-8879

Vladimir M. Shipulin – Dr. Sc. (Med.), Professor, Honored Scientist of the Russian Federation, Chief Research Officer, Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences, e-mail: shipulin@cardio-tomsk.ru, https://orcid.org/0000-0003-1956-0692

Irina V. Sukhodolo – Dr. Sc. (Med.), Professor, Professor at the Department of Morphology and General Pathology, Siberian State Medical University, e-mail: suhodolo.iv@ssmu.ru, https://orcid.org/0000-0001-9848-2068

Angelina I. Stelmashenko – Teaching Assistant at the Department of Morphology and General Pathology, Siberian State Medical University, e-mail: staranie@mail.ru, https://orcid.org/0000-0002-8284-6864

Olga A. Denisenko – External Doctorate Student at the Department of Pathophysiology, Siberian State Medical University, e-mail: olga-muraveinik@yandex.ru, https://orcid.org/0000-0003-4524-8491

Sergey L. Andreev – Cand. Sc. (Med.), Research Officer, Cardiovascular Surgeon at the Cardiosurgical Department No. 1, Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences, e-mail: anselen@rambler.ru, https://orcid.org/0000-0003-4049-8715
Maksim S. Demin – Postgraduate at the Department of Pathophysiology, Siberian State Medical University, e-mail: maksimd25111996@mail.ru, https://orcid.org/0009-0001-0951-3158
Elena G. Churina – Dr. Sc. (Med.), Professor at the Department of Pathophysiology, Siberian State Medical University; Professor at the Department of Organic Chemistry, National Research Tomsk State University, e-mail: Lena1236@yandex.ru, https://orcid.org/0000-0002-8509-9921

MICROBIOLOGY AND VIROLOGY

GENETIC HETEROGENEITY OF *RICKETTSIA HELVETICA* POPULATION

Rar V.A. [1],
Igolkina Ya.P. [1],
Yakimenko V.V. [2],
Tikunov A.Yu. [1],
Nikitin A.Ya. [3],
Epikhina T.I. [1],
Tikunova N.V. [1]

[1] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (Lavrentyeva Ave. 8, Novosibirsk 630090, Russian Federation)
[2] Omsk Research Institute of Natural Focal Infections (Mira Ave. 7, Omsk 644080, Russian Federation)
[3] Irkutsk Antiplague Research Institute of Siberia and Far East of Rospotrebnadzor (Trilissera str. 78, Irkutsk 664047, Russian Federation)

Corresponding author:
Vera A. Rar,
e-mail: rarv@niboch.nsc.ru

ABSTRACT

Background. *To date, the genetic variability of Rickettsia helvetica has not been sufficiently studied.*
The aim. *To study the prevalence and genetic variability of R. helvetica in Ixodes spp. collected in Western Siberia and the Russian Far East.*
Materials and methods. *Ixodes spp. collected from rodents in the Omsk province, Western Siberia (n = 280) and collected by flagging on Putyatin and Russky Islands in Primorsky Krai, Russian Far East (n = 482) were analyzed for the presence of Rickettsia spp. All positive samples were genotyped for the gltA gene fragment. For a number of R. helvetica samples, fragments of the 16S rRNA, ompA, ompB, sca4, htrA, and groEL genes and 23S–5S intergenic spacer were additionally sequenced.*
Results. *Four Rickettsia species (R. helvetica, "Candidatus Rickettsia tarasevichiae", "Candidatus Rickettsia uralica", and "Candidatus Rickettsia mendelii") were found. Of them, R. helvetica was identified in 72.2 % of Ixodes apronophorus and 18.8 % of Ixodes trianguliceps from the Omsk province and in single Ixodes persulcatus from the Omsk province and Putyatin Island. This is the first finding of Rickettsia spp. in I. apronophorus. All known R. helvetica sequences from this study and the GenBank database belonged to four well supported monopheletic groups forming genetic lineages I–IV. Lineage I included European isolates from Ixodes ricinus, Western Siberian isolates from I. persulcatus, and some sequences from I. apronophorus. All R. helvetica sequences from I. trianguliceps from the Omsk province and I. persulcatus from the Komi Republic and one sequence from I. apronophorus were assigned to lineage II. Most sequences from I. apronophorus formed lineage III; all known R. helvetica sequences from I. persulcatus from the Far East formed genetic lineage IV.*
Conclusion. *The genetic heterogeneity of R. helvetica population was first demonstrated. Known isolates of R. helvetica are reliably assigned to four genetic lineages, but not in all cases association of different lineages with a specific tick species or specific territory was observed.*

Key words: *Ixodes apronophorus, Ixodes persulcatus, Ixodes trianguliceps, Rickettsia helvetica, sympatric areas, genetic lineages, phylogenetic analysis*

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ГЕНЕТИЧЕСКАЯ ГЕТЕРОГЕННОСТЬ ПОПУЛЯЦИИ *RICKETTSIA HELVETICA*

Рар В.А. [1],
Иголкина Я.П. [1],
Якименко В.В. [2],
Тикунов А.Ю. [1],
Никитин А.Я. [3],
Епихина Т.И. [1],
Тикунова Н.В. [1]

[1] ФГБУН Институт химической биологии и фундаментальной медицины СО РАН (630090, г. Новосибирск, просп. Лаврентьева, 8, Россия)
[2] ФБУН «Омский научно-исследовательский институт природно-очаговых инфекций» (644080, г. Омск, просп. Мира, 7, Россия)
[3] ФКУЗ Иркутский научно-исследовательский противочумный институт Сибири и Дальнего Востока Федеральной службы по надзору в сфере защиты прав потребителей и благополучия человека (664047, г. Иркутск, ул. Трилиссера, 78, Россия)

Автор, ответственный за переписку:
Рар Вера Александровна,
e-mail: rarv@niboch.nsc.ru

РЕЗЮМЕ

Обоснование. *Генетическая вариабельность Rickettsia helvetica недостаточно изучена.*
Цель исследования. *Изучить встречаемость и генетическую вариабельность R. helvetica в Ixodes spp., собранных в Сибири и на Дальнем Востоке.*
Методы. *На наличие риккетсий проанализированы клещи, снятые с грызунов в Омской области (n = 280) и собранные на флаг на островах Путятина и Русский в Приморском крае (n = 482). Для всех образцов риккетсий секвенированы фрагмента гена gltA, а для ряда образцов R. helvetica дополнительно секвенированы фрагменты 16S rRNA, ompA, ompB, sca4, htrA и groEL генов и 23S–5S межгенного спейсера.*
Результаты. *Всего было выявлено четыре вида риккетсий. Из них R. helvetica обнаружена в 72,2 % Ixodes apronophorus и 18,8 % Ixodes trianguliceps из Омской области и в единичных Ixodes persulcatus из Омской области и с острова Путятина. Это первое выявление риккетсий в I. apronophorus. На основании проведённого филогенетического анализа последовательности R. helvetica из данной работы и из базы данных GenBank отнесены к четырём генетическим линиям. Линия I включает европейские изоляты из Ixodes ricinus, изоляты из I. persulcatus из Западной Сибири и некоторые последовательности из I. apronophorus. Все последовательности R. helvetica из I. trianguliceps из Омской области и из I. persulcatus из Республики Коми, а также последовательность из I. apronophorus отнесены к линии II. Большинство последовательностей из I. apronophorus образуют линию III, а все последовательности R. helvetica из I. persulcatus с Дальнего Востока – линию IV.*
Заключение. *Впервые показана генетическая гетерогенность популяции R. helvetica. Известные изоляты R. helvetica надёжно отнесены к четырём генетическим линиям, однако ассоциация различных линий с определённым видом клеща или с определённой территорией наблюдается не во всех случаях.*

Ключевые слова: *Ixodes apronophorus, Ixodes persulcatus, Ixodes trianguliceps, Rickettsia helvetica, область симпатрии, генетические линии, филогенетический анализ*

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INTRODUCTION

Rickettsia helvetica Beati et al. 1993 [Rickettsiaceae; Rickettsiales], belonging to the spotted fever group (SFG), is one of the causative agents of rickettsioses in Eurasia. Rickettsiosis caused by *R. helvetica* is characterized by a wide range of clinical manifestations: fever is more often without rash, and cases of perimyocarditis, meningitis, and sarcoidosis have been described [1–5]. This infection is mainly diagnosed in European countries. In Russia, only one case of rickettsiosis caused by *R. helvetic*a has been described in a patient with signs of acute febrile illness from the Perm Territory [6].

Specific vectors of *R. helvetica* are ticks of the genus *Ixodes*. *Rickettsia helvetica* is widely prevalent in ticks *Ixodes ricinus* (Linnaeus, 1758) in various European countries [7, 8]. In Russia, *R. helvetica* is observed in different regions; however, in most regions the occurrence of this species was rather low and did not exceed 5–8 %. Thus, *R. helvetica* was found in 4.6 % of the taiga tick (*Ixodes persulcatus* (Schulze, 1930)) in the Komi Republic [9], in 1.9 % of *I. persulcatus* in the Omsk region [10], in 5.1 % of *Ixodes* spp. in the Altai Territory [11], as well as in 8.1 % of *Ixodes persulcatus* (Pomerantzev, 1946) and in 6.9 % of interspecies hybrids of *I. persulcatus*/*I. pavlovskyi* in the Altai Republic [12]. In the mainland of the Far East, the occurrence of *R. helvetica* in taiga ticks was also low and amounted to 3.8–4.3 % in some areas of Khabarovsk Territory, and 2.4 % in the south of the Kamchatka Peninsula [13, 14]. The exception is Sakhalin Island, where the occurrence of *R. helvetica* in *I. persulcatus* exceeded 60 % [14].

In addition to *I. persulcatus* and *I. pavlovskyi* attacking humans, the nidicolous ticks *Ixodes trianguliceps* (Birula, 1895) and Ixodes *apronophorus* (Schulze, 1924), all developmental stages of which feed on small mammals, inhabit various areas of Western Siberia. Both species have an extensive but mosaic range. The tick *I. apronophorus* is a moisture-loving species inhabiting wetlands; one of its main hosts is the European water vole (*Arvicola amphibius* (Linnaeus, 1758)) [15–18].

Many works have been devoted to the study of *I. persulcatus* for the presence of various species of rickettsia; most often, "*Candidatus* Rickettsia tarasevichiae" is detected in *I. persulcatus* ticks (up to 90 % of infected ticks), and in rare cases – *R. helvetica*, *Rickettsia heilongjiangensis*, *Rickettsia raoultii* and *Rickettsia sibirica* [10, 12, 14, 19]. The tick *I. trianguliceps* is significantly less studied; in our previous studies, a new candidate species "*Candidatus* Rickettsia uralica" was found in *I. trianguliceps* ticks, as well as *R. helvetica* and "*Candidatus* R. tarasevichiae" [19]. Data on the infection of *I. apronophorus* ticks with rickettsiae were not available at the beginning of this study.

In the southern taiga and sub-taiga of Western Siberia there are areas of sympatry of three tick species that belong to the genus *Ixodes*: *I. persulcatus*, *I. trianguliceps* and *I. apronophorus*. In these areas of sympatry, the preimaginal stages of *I. persulcatus* and all developmental stages of *I. trianguliceps* and *I. apronophorus* can feed on the same small mammals, which may result in the transmission of any rickettsial species/gene variants from one tick species to another. In preliminary studies, we have discovered sites in the sympatric regions of *I. apronophorus*/*I. persulcatus*/*I. trianguliceps* in the Omsk region with the high abundance of all three tick species [20]. Islands in the Far East are also of great interest for study, as one of these islands (Sakhalin Island) had an unexpectedly high infection rate of the taiga tick with *R. helvetica* [14].

THE AIM OF THE STUDY

To study the occurrence and genetic variability of *R. helvetica* in different species of ticks of the genus *Ixodes* in remote areas of the Omsk region and the Far East.

MATERIALS AND METHODS

Collecting ticks

The material was collected on the territory of Bolsheukovsky district (site Om-Bo, 56° 46′ N, 72° 03′ E) and Znamensky district (site Om-Zn, 57° 23′ N, 73° 40′ E) of the Omsk Region, as well as on the territory of Putyatin Island (site Put 42° 50′ N, 132° 25′ E) and Russky Island (site Rus 43° 00′ N, 131° 50′ E), located in Peter the Great Bay of the Sea of Japan in Primorsky Territory (Fig. 1). *Ixodes* spp. ticks collected from rodents in the Omsk region and ticks collected by flagging in Primorsky Territory were included in the study.

Rodents were captured in the Omsk region at the Om-Bo site in June 2016 and at the Om-Zn site from June through September 2014–2015. Animals were examined for the presence of attached ticks (larvae, nymphs and adults), which were removed with tweezers. The species and developmental stage of ticks were preliminarily determined using a stereomicroscope MC-800 (Micros, Austria), according to morphological keys [21]. Some of the engorged and nearly engorged larvae and nymphs were stored at 10–15 °C for 1–2 weeks and then transported to the laboratory for metamorphosis. The remaining ticks were placed in sealed plastic tubes that were stored in liquid nitrogen until DNA extraction.

Questing ticks were collected from vegetation by flagging on Putyatin Island in 2021 and on Russky Island in 2019 and 2021. The species and developmental stage of ticks were preliminarily determined based on morphological criteria; only ticks of the genus *Ixodes* were included in further study.

Carrying out metamorphosis of ticks under laboratory conditions

Partially engorged larvae and nymphs were fed on laboratory white mice to repletion to successfully undergo metamorphosis. Each engorged tick was placed in an individual glass tube and kept in the dark at 100 % relative humidity at 24–26 °C until moulting was completed. The ticks that underwent metamorphosis after 4 weeks were individually frozen and stored at –70 °C until DNA isolation.

FIG. 1.
Tick collection sites

DNA isolation

Frozen ticks were homogenized with a MagNA Lyser Instrument using MagNa Lyser Green Beads (Roche Diagnostics, Switzerland). Total DNA was isolated using the Proba NK kit (DNA-Technology, Russia) according to the manufacturer's protocol.

Determination of tick species by molecular genetic methods

The species of *I. persulcatus*, *I. trianguliceps*, and *I. apronophorus* ticks was determined by multiplex polymerase chain reaction (PCR) using species-specific primers for the ITS2 intergenic spacer fragment as previously described [20]. *I. persulcatus*, *I. pavlovskyi* and interspecific hybrids were differentiated based on the determination of mitochondrial (cox1) and nuclear (ITS2) loci as described previously [12].

Identification and genotyping of *Rickettsia helvetica*

Rickettsia DNA was detected using nested PCR in the presence of genus-specific primers from the *glt*A gene region in the first round and primers specific for "*Candidatus* R. tarasevichiae" and specific for SFG rickettsiae in the second round, as described previously [14]. The obtained *glt*A gene fragments were sequenced for all positive rickettsiae samples from the SFG. For a number of positive samples containing *R. helvetica* DNA, gene fragments of 16S rRNA, *omp*A, *omp*B, *sca*4, and *htr*A, as well as a fragment of the *gro*ESL operon and the intergenic spacer 23S-5S rRNA (23S-5S IGS) were additionally amplified in the presence of primers indicated in Table 1 for subsequent sequencing. In addition, the same fragments of seven genetic loci were amplified for samples of *R. helvetica* revealed earlier on Sakhalin Island (site Skh) and in Khabarovsk Territory (site Khab) [14]. The identified nucleotide sequences have been deposited in the GenBank database under accession numbers OQ092468-OQ092487, OQ102487-OQ102493, OQ271213-OQ271221, OQ275007-OQ275011, OQ675828-OQ675832, OQ861252, OQ866612-OQ866624.

Sequencing and phylogenetic analysis

PCR products were purified using GFX columns (Amersham Biosciences, USA). The Sanger sequencing reaction was performed using BigDye Terminator v. 3.1 Cycling Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequencing products were purified using CentriSep columns (Princeton Separations, USA) and analyzed using ABI 3500 Genetic Analyzer (Applied Biosystems, USA). Sequence analysis was performed using BlastN [29]. Phylogenetic analysis was performed with the MEGA 7.0 phylogenetic software package [30] using the maximum likelihood (ML) method [31].

RESULTS

Species identification of collected ticks

The species identity of all ixodid ticks collected in the Omsk Region and Primorsky Territory was determined using molecular genetic methods. Ticks of three species (*I. apronophorus*, *I. persulcatus* and *I. trianguliceps*) were found in two sites in the Omsk region, but the pro-

TABLE 1

PRIMERS USED FOR *R. HELVETICA* AMPLIFICATION

Locus	Organism	Round	Primer	Sequence 5′-3′	T (°C)	Ref.
16S gene rRNA	*Rickettsia* spp.	I	16S1	gacgggtgagtaacacgtggg	56	[14]
			16S2	gtcttttagggatttgctccac		
		II	16S3	gatggatgagcccgcgtcag	60	
			16S4	gcatctctgcgatccgcgac		
ompA gene Fragment I	*Rickettsia* spp.		Rr190.70p	atggcgaatatttctccaaaa	55	[22]
			190-701	gttccgttaatggcagcatct		
ompA gene	*Rickettsia* spp.	I	Afnw_1	ggcacaaatactttaacattacc	52	#
			190-6808	cacgaactttcacactacc		[22]
		II	Afnw_3	aagcctactcctaaagagaatg	53	#
			Afnw_4	cgacagtctctagtgccg		
	Rickettsia spp.	I	A1	taacattacaagctggaggaagcc	58	#
			A2	ttcagagcctgaccaccgg		
		II	A5	caagtgctggtgatgttacta	56	
			A6	tagttacatttcctgcacctac		
	R. helvetica	I	Afn1_helv	gtaatactagcatcaccgaaatcc	55	#
			190-6808	cacgaactttcacactacc		[22]
		II	190-5125	gcggttactttagccaaagg	54	
			Afnw_4	cgacagtctctagtgccg		#
ompB gene	*Rickettsia* spp.	I	M59 F	ccgcagggttggtaactgc	55	[23]
			120-1497m*	cctatatcgccggtaattgtagc		
		II	BR1	gttactaatggatttattcaagt	53	#
			BR2	gcataaacttgtccagcgat		
	Rickettsia spp.	I	B2f_5	taaacttgctgacggtacag	56	#
			B2f_2	cgattatgccgttatcgcttccaag		
		II	B2f_3	gtagcctaacaaatgctcaaac	52	
			120-2399	cttgtttgtttaatgttacggt		
	R. helvetica	I	B2f_3	gtagcctaacaaatgctcaaac	52	[23]
			B2f_2	cgattatgccgttatcgcttccaag		
		II	B2f_1helv	cagtacaattcgctcacaacac	55	#
			120-2399	cttgtttgtttaatgttacggt		
	Rickettsia spp.	I	B1	atatgcaggtatcggtact	56	#
			B2	ccatataccgtaagctacat		
		II	B3	gcaggtatcggtactataaac	56	
			B4	aatttacgaaacgattacttccgg		

TABLE 1 (*continued*)

	Rickettsia spp.	I	120-3462	ccacaggaactacaaccatt	52	[23]
			120-4879m*	tagaagtttacacggacttttagag		
		II	B3f_3f	gctggacctgaagctggagc	55	#
			120-4879m*	tagaagtttacacggacttttagag		[23]
*sca*4 gene	*Rickettsia* spp.	I	D1f	atgagtaaagacggtaacct	52	[24]
			D1876rm*	tagtttgttccgccgtaatc		
		II	sc1f_3	gatgtaggtgatgaactctg	52	#
			D1390r	cttgcttttcagcaatatcac		[24]
		I	sc4-1	atgtctctgaattaagcaatgc	52	#
			Rj2837r	cctgatactacccttacatc		[25]
		II	sc4-5	ccggcacaacaacaattgatg	50	#
			sc4-6	cctttaccagctcatctactt		
		I	sc4-3	aattattaggctctgtattaaaga	52	#
			D3069r	tcagcgttgtggaggggaag		[24]
		II	sc4-5	ccggcacaacaacaattgatg	52	#
			sc4-7	ctctcttttaataggtgttgatt		#
*htr*A gene	*Rickettsia* spp.		17k-5	gctttacaaaattctaaaaaccatata	55	[26]
			17k-3	tgtctatcaattcacaacttgcc		
23S-5S IGS	*Rickettsia* spp.		RCK/23-5-F	gataggtcrgrtgtggaagca	55	[27]
			RCK/23-5-R	tcgggaygggatcgtgtgtttc		
groESL Operon	*Rickettsia* spp.	I	Ric-ESL-F1	ggtaaatgggcaggyaccgaa	60	[28]
			Ric-ESL-R1	gaagcaacrgaagcagcatctt		
		II	Ric-ESL-F2	atcgttatgaaagaaagcgayg	58	
			Ric-ESL-R2	agwgcagtacgcactactttagc		

Note. T (°C) – annealing temperature; m* – modified primer; # – this study.

portion of ticks of different species on these sites differed significantly. At the Om-Bo site, 67 (40.6 %) *I. apronophorus*, 73 (44.2 %) *I. persulcatus* and 25 (15.1 %) *I. trianguliceps* were identified among 145 ticks removed from 29 rodents and 20 ticks removed from rodents and underwent metamorphosis in laboratory conditions, and at the Om-Zn site among 115 ticks removed from rodents and molted in the laboratory, 5 (4.4 %) *I. apronophorus*, 87 (75.6 %) *I. persulcatus* and 23 (20.0 %) *I. trianguliceps* were found (Table 2).

In the Far East, on Putyatina Island, 56 *I. persulcatus,* 4 *I. pavlovskyi* and one *I. persulcatus*/*I. pavlovskyi* interspecies hybrid were identified among unfed adults collected from vegetation by flagging, and on Russian Island – 190 *I. persulcatus,* 199 *I. pavlovskyi* and 32 interspecies hybrids were found (Table 2).

Identification of *Rickettsia* spp. in *Ixodes* spp. ticks in the Omsk region

Among ticks from the Om-Bo site collected from rodents but not molted *R. helvetica* DNA was identified in 45 (72.5 %) *I. apronophorus* of all development stages (including 1 case of *R. helvetica* and "*Candidatus* R. tarasevichiae" mixed infection) and in 9 (37.5 %) *I. trianguliceps* but was not identified in *I. persulcatus.* Notably, all infected *I. trianguliceps* were larvae (Table 2). Additionally, "*Candidatus* R. tarasevichiae" DNA was found in 48 (81.4 %) *I. persulcatus* and 1 (1.6 %) *I. apronophorus* larvae, and "*Candidatus* R. uralica" DNA was found in 7 (29.2 %) *I. trianguliceps*. "*Candidatus* R. tarasevichiae" with *Rickettsia raoultii* was detected in one larva (1.6 %) of *I. persulcatus*.

Among the molted ticks, 3 out of 5 are *I. apronophorus* from the Om-Bo site and 4 of 5 *I. apronophorus*

TABLE 2

IDENTIFICATION OF *R. HELVETICA* IN *IXODES* SPP. TICKS

Sites/ analyzed ticks	Tick species	Stage	Tick population	Ticks containing *R. helvetica* DNA, abs. (%)
Om-Bo/ collected from rodents	*I. apronophorus*	Larvae	47	33
		Nymphs	5	4
		Adults	10	8
		All stages	**62**	**45 (72.5)**
	I. persulcatus	Larvae	55	0
		Nymphs	4	0
		All stages	**59**	**0**
	I. trianguliceps	Larvae	17	9
		Nymphs	4	0
		Adults	3	0
		All stages	**24**	**9 (37.5)**
Om-Bo/ molted	*I. apronophorus*	Nymphs	2	1
		Adults	3	2
		All stages	**5**	**3 (60)**
	I. persulcatus	Nymphs	3	0
		Adults	11	0
		All stages	**14**	**0**
	I. trianguliceps	Nymphs	1	0
		All stages	**1**	**0**
Om-Zn/ molting	*I. apronophorus*	Nymphs	4	3
		Adults	1	1
		All stages	**5**	**4 (80)**
	I. persulcatus	Nymphs	23	0
		Adults	64	1
		All stages	**87**	**1 (1.1)**
	I. trianguliceps	Nymphs	10	0
		Adults	13	0
		All stages	**23**	**0**

TABLE 2 (*continued*)

Put/ collected by flagging	*I. persulcatus*	**Adults**	**56**	**1 (1.8)**
	I. pavlovskyi	**Adults**	**4**	**0**
	hybrids	**Adults**	**1**	**0**
Rus/ collected by flagging	*I. persulcatus*	**Adults**	**190**	**0**
	I. pavlovskyi	**Adults**	**199**	**0**
	hybrids	**Adults**	**32**	**0**

Note. Combined data for all stages examined for each tick species are shown in bold.

from the Om-Zn site contained *R. helvetica* DNA. In addition, *R. helvetica* was revealed in one molted *I. persulcatus* as a mixed infection with "*Candidatus* R. tarasevichiae" (Table 2). "*Candidatus* R. tarasevichiae" DNA was revealed in 80 (79.2 %) *I. persulcatus* and 2 (8.3 %) *I. trianguliceps*, while "*Candidatus* R. uralica" DNA was detected in 3 (12.5 %) *I. trianguliceps*. A mixed infection "*Candidatus* R. uralica" with *Rickettsia* sp. was revealed in 1 (1 %) nymph *of I. persulcatus*.

Combining data obtained for all examined ticks from the Omsk region, *R. helvetica* was revealed in 75.0 % of *I. apronophorus*, 18.8 % of *I. trianguliceps* and 1.9 % of *I. persulcatus*, indicating a close ecological relationship between *R. helvetica* and *I. apronophorus*.

Identification of *Rickettsia spp.* in *Ixodes* spp. ticks in the Primorsky Territory

R. helvetica DNA was revealed in only 1 of 56 *I. persulcatus* from Putyatin Island, but was not revealed in any other tick species from the same site, nor in any of 421 ticks of the genus *Ixodes* from Russky Island (Table 2). "*Candidatus* R. tarasevichiae" DNA was revealed in 42 (75.0 %) *I. persulcatus*, in 1 of 4 *I. pavlovskyi* and in the single interspecies hybrid from Putyatin Island, and in 73.7 % of *I. persulcatus*, 5.3 % of *I. pavlovskyi* and 31.3 % of inter-species hybrids from Russky Island. DNA of "*Candidatus* Rickettsia mendelii" was revealed in 2 *I. pavlovskyi* on Russky Island (2016).

R. helvetica genotyping

Sequences of the *glt*A gene fragment (840 nucleotide pairs) were determined for all *R. helvetica* isolates, and 6 sequence variants were identified based on analysis of these sequences. Fragments of *omp*B (1255 bp), *sca*4(783 bp), and 16S rRNA (684 bp) genes were additionally sequenced for a number of samples with different variants of the *glt*A gene. Comparative analysis of the sequences obtained revealed 7 sequence variants of *R. helvetica* differing from each other by 2–8 nucleotide substitutions; all detected sequence variants differed from the sequence of the prototypical strain C9P9 (AICO01000001) (Fig. 2a).

Phylogenetic analysis based on comparison of the concatenated sequences *glt*A-*omp*B-*sca*4 with a total length of 2259 bps showed that the sequences obtained belong to four genetic lineages (Fig. 3). The samples belonging to lineage I (European lineage) formed a common cluster together with the prototype strain *R. helvetica* C9P9 isolated from the tick *I. ricinus* from Switzerland. Lineage I sequences were revealed only at the Om-Zn site in three *I. apronophorus* and one *I. persulcatus* (Table 3); these sequences differed from the *R. helvetica* C9P9 sequences by single substitutions at the *gltA* or *ompB* genes (Fig. 2a). Lineage II (*I. trianguliceps* line) included all samples of *R. helvetica* from *I. trianguliceps* ticks and one sample from molted *I. apronophorus* from the Om-Bo site. The lineage II sequences determined in this study were identical to previously determined sequences from two nymphs of *I. trianguliceps* taken from rodents from another area of Omsk region. Lineage III (*I. apronophorus* line) was the most numerous and included sequences of 48 *I. apronophorus*, mainly from the Om-Bo site. Samples from this lineage were genetically heterogeneous – one sample differed by a single substitution in the *glt*A gene from the others; notably, 5 tick samples taken from the same rodent had the same substitution in the 16S rRNA gene. Lineage IV (Far Eastern lineage) included all determined sequences of *R. helvetica* from *I. persulcatus* from the Far East: Putyatin Islands, Sakhalin and Khabarovsk Territory (Table 3).

Since most *R. helvetica* isolates from the GenBank database have been characterized using only the *omp*B gene, we used this locus to compare the *R. helvetica* sequences obtained in this study with those published previously. Phylogenetic analysis based on comparison of the sequences of the *omp*B gene, 2684 bps in length, showed the presence of the same four genetic lineages. Based on the analyses conducted, the European lineage (lineage I) additionally contained a number of specimens of *R. helvetica* from *I. ricinus* originated from Germany and one specimen from *I. persulcatus* from the Novosibirsk region, and the *I. trianguliceps* linage (lineage II) additionally included 32 specimens of *R. helvetica* from *I. persulcatus* from the Komi Republic (Fig. 4). Sequence analy-

Isolates	Tick species	Region	Lineage	*gltA*					*ompB*					*sca4*		*16S*
				175525	175576	175847	176092	176156	381079	381549	381958	382176	382239	812583	812147	523727
C9P9	*I. ricinus*	Europe	I	G	C	T	C	C	G	C	G	G	G	G	C	T
Om-74_Iapr_m	*I. apronophorus*	Siberia	I	.	.	.	.	.	.	.	.	T	.	.	.	.
Om-20_Iper_m	*I. persulcatus*	Siberia	I	.	.	.	T	.	nd	nd	nd	nd	nd	.	.	nd
Om-75_Itr	*I. apronophorus*	Siberia	II	A	.	.	.	T	A	.	A	T	.	.	.	.
Om-145_Iapr	*I. apronophorus*	Siberia	III	.	T	.	.	.	.	T	A	T	A	.	T	.
Om-79_Iapr	*I. apronophorus*	Siberia	III	.	T	.	.	.	.	T	A	T	A	.	T	G
Om-103_Iapr	*I. apronophorus*	Siberia	III	.	T	A	nd	nd	.	T	nd	nd	nd	.	T	nd
Skh-7_Iper	*I. persulcatus*	Far East	IV	.	.	.	.	.	.	.	A	T	.	A	.	.
Put-117_Iper	*I. persulcatus*	Far East	IV	.	.	.	.	.	.	.	A	T	.	A	.	.

a

Isolates	Lineage	*gltA*			*ompA*			*ompB*												*sca4*						*htrA*	*16S*		*IGS*	
		175525	175576	176156	209675	208648	208557	380060	380249	380592	380622	381079	381549	381958	382176	382239	382562	382824	382895	383024	813613	813472	813341	812583	812147	811867	195041	523727	523907	1187522	1187463
C9P9	I	G	C	C	C	G	T	T	A	C	T	G	C	G	G	G	G	T	C	G	A	A	C	G	C	G	T	T	A	T	G
Novosibirsk08-5	I	.	.	.	nd	nd	nd	.	.	.	.	.	.	.	.	.	.	.	.	.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Komi	II	.	.	T	nd	nd	nd	.	G	.	C	A	.	A	T	.	A	.	.	.	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Om-74_Iapr_m	I	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Om-75_Itr	II	A	.	T	T	.	.	.	G	T	C	A	.	A	T	.	A	.	.	.	C	G	.	.	.	A	.	.	.	C	.
Om-145_Iapr	III	.	T	.	.	.	.	G	.	.	C	.	T	A	T	A	.	C	T	.	.	.	.	.	T	.	G	.	G	.	.
Om-79_Iapr	III	.	T	.	.	.	.	G	.	.	C	.	T	A	T	A	.	C	T	.	.	.	.	.	T	.	G	G	G	.	.
Skh-7_Iper	IV	.	.	.	.	A	C	.	.	.	C	.	.	A	T	.	.	C	.	.	.	.	T	A	.	.	.	.	.	C	A
Put-117_Iper	IV	.	.	.	.	A	.	.	.	.	C	.	.	A	T	.	.	C	.	A	.	.	T	A	.	.	.	.	.	C	A

b

FIG. 2.

a *– condensed alignment based on gene sequences of gltA (840 bp), ompB (1255 bp), sca4 (783 bp) and 16S rRNA (684 bp) sequence variants of R. helvetica;* ***b*** *– condensed alignment based on gene sequences gltA (1037 bp), ompA (1417 bp), ompB (3100 bp), sca4 (2398 bp), htrA (499 bp), 16S rRNA (1070 bp) genes and the intergenic spacer 23S-5S rRNA (23S-5S IGS) (489 bp) of R. helvetica genetic lines. Polymorphic sites are listed according to the sequence of R. helvetica, strain C9P9 (AICO01000000). Non-synonymous substitutions are marked in green color*

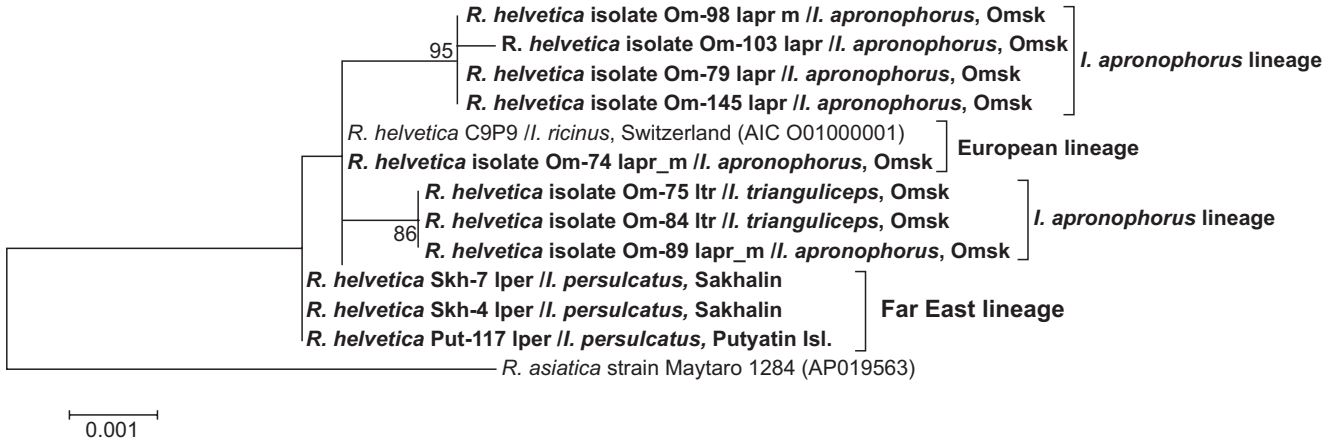

FIG. 3.

Dendrogram constructed by ML method based on concatenated sequences of gltA-ompB-sca4 gene fragments (2259 bps). Sequences obtained in this study are highlighted in bold font

TABLE 3

PREVALENCE OF DIFFERENT GENETIC LINEAGES OF *R. HELVETICA* IN *IXODES* SPP.

Site	Tick species	Analyzed ticks	Number of genotyped *R. helvetica* samples	Number of *R. helvetica* specimens belonging to the lineage			
				I	II	III	IV
Om-Bo	*I. apronophorus*	Not molted	45	0	0	45	0
		Molted	3	0	1	2	0
		Total	**48**	**0**	**1**	**47**	**0**
	I. trianguliceps	Not molted	9	0	9	0	0
Om-Zn	*I. apronophorus*	Molted	4	3	0	1	0
	I. persulcatus	Molted	1	1	0	0	0
Put	*I. persulcatus*	Collected by flagging	1	0	0	0	1
Khab	*I. persulcatus*	Collected by flagging	1	0	0	0	1
Skh	*I. persulcatus*	Collected by flagging	4	0	0	0	4

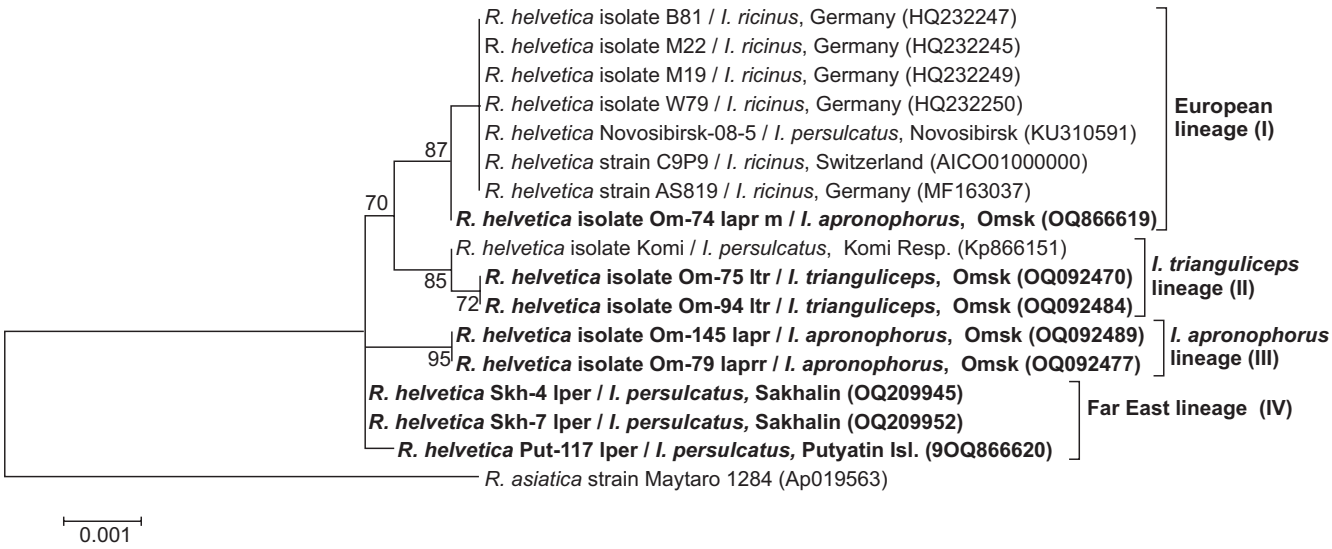

FIG. 4.

Dendrogram constructed by ML method based on sequences of the ompB gene fragment (2684 bps) of R. helvetica. R. asiatica sequence was used as an outgroup. Sequences obtained in this study are highlighted in bold font

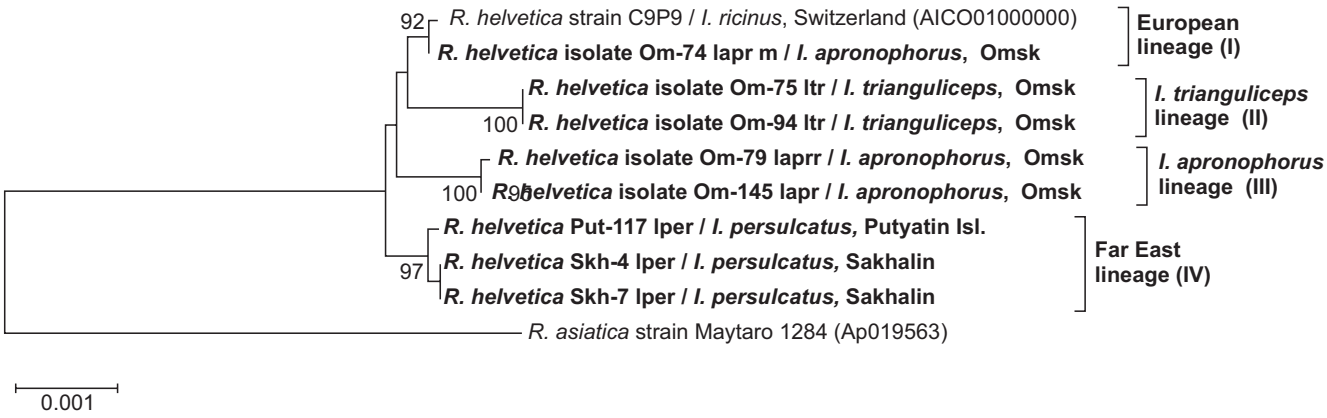

FIG. 5.

A dendrogram constructed by the ML method based on concatenated sequences of fragments of seven loci (gltA – ompA – ompB – sca4 – htrA – 16S rRNA – IGS) (9840 bp). Sequences obtained in this study are highlighted in bold font

sis of the *sca*4 gene revealed that the Far Eastern lineage also includes *R. helvetica* specimens from *I. persulcatus* from Japan [25].

For more detailed genotyping of the 16S rRNA gene sequence (1070 bp), *glt*A (1037 bp), *omp*A (1417 bp), *omp*B (3100 bp), *sca*4 (2398 bp), *htr*A (499 bp), *GroEL* (1528 bp), as well as 23S-5S IGS (489 bp) were determined for eight samples of *R. helvetica* belonging to different genetic lineages. All *gro*EL gene sequences were identical. The remaining genetic loci had polymorphic sites; of these, the *omp*B gene was the most variable. Among the coding sequences, nucleotide substitutions at 15 of the 25 polymorphic sites were nonsynonymous (Fig. 2b). Phylogenetic analysis based on a comparison of the concatenated sequences 16S – *glt*A – *omp*A – *omp*B – *sca*4 – *htr*A – IGS (9840 bps) also revealed with a high level of support the presence of four clusters that corresponded to the genetic lineages identified based on the analysis of shorter sequences (Fig. 5). It should be noted that within each genetic lineage, *R. helvetica* samples differed among themselves by 1–2 nucleotide substitutions. In the case of genetic lines I and II, samples from the Omsk region differed from samples from other regions, and in the case of genetic lineage IV, the sample from Putyatin Island differed by two substitutions from samples from Sakhalin.

DISCUSSION

Different species of rickettsiae tend to be associated with certain species of ticks. *Rickettsia helvetica* is closely related to ticks of the genus *Ixodes* and is the dominant species of rickettsia in *I. ricinus* in Europe and *I. persulcatus* in some regions of Russia (Sakhalin Island and Komi Republic) [7, 9, 14].

The study of tick infectivity with infectious agents in areas of sympatry is of particular interest because it allows us to compare the pathogen-tick association for different tick species in the same area. This study included ticks collected at two sites in the areas of sympatry *I. apronophorus*/*I. persulcatus*/*I. trianguliceps* in the Omsk region; at the Om-Bo site the abundance of all three tick species was high, while at the Om-Zn site *I. persulcatus* was dominant and the abundance of *I. apronophorus* was low. In this study, rickettsiae were first revealed in *I. apronophorus*. *Rickettsia helvetica* was revealed in 60–80 % of ticks at different developmental stages from both sites, indicating a close association of *R. helvetica* with *I. apronophorus* (Table 2).

In the Omsk region, *R. helvetica* was also revealed in single *I. persulcatus* and in 38 % of *I. trianguliceps* collected from rodents from the Om-Bo site. It should be noted that *R. helvetica* was revealed only in *I. trianguliceps* larvae, but not in nymphs and adults (Table 2). Considering that all larvae infected with *R. helvetica* were collected from only two voles, the observed discrepancy can be explained by the insufficient number of *I. trianguliceps* examined and the uneven distribution of infected and uninfected larvae, being offspring from different females. This uneven distribution of larvae can also explain the fact that all *R. helvetica* samples with a unique substitution in the 16S rRNA gene were revealed only in larvae (but not in adults) of *I. apronophorus* collected from the same vole.

In addition to the areas of sympatry in the Omsk region, ticks collected on two islands in Primorsky Territory were also included in the study. Since an unexpectedly high level of infection of taiga ticks with *R. helvetica* was previously observed on Sakhalin Island, it could be expected that other islands would also show an atypical distribution of *Rickettsia* spp. in different tick species. Notwithstanding, on the surveyed Putyatin and Russky islands, as well as on the mainland of the Far East and in Western Siberia [10, 12–14, 19], "*Candidatus* R. tarasevichiae" was significantly dominant in *I. persulcatus* ticks, and *R. helvetica* was revealed only in one *I. persulcatus* on Putyatin Island (Table 2).

Rickettsia helvetica is a highly variable species. Analysis of the sequences of this species determined in this study

and available in the GenBank database resulted in the assignment of *R. helvetica* isolates to four genetic lineages, but the association of different lineages with a particular tick species or territory was not observed in all cases. Thus, genetic lineage I combined all genotyped specimens from *I. ricinus* from Europe, a number of specimens from *I. apronophorus* from the Omsk region and specimens from *I. persulcatus* from the Omsk and Novosibirsk regions. The genetic lineage of *I. trianguliceps* (lineage II) was found in two different species of *Ixodes* spp. in distant regions: in *I. trianguliceps* in the Omsk region and *I. persulcatus* in the Komi Republic. At the same time, the genetic lineage of *I. apronophorus* (lineage III) was revealed only in *I. apronophorus* in the Omsk region; and the Far Eastern genetic lineage (lineage IV) was revealed only in *I. persulcatus* in the Far East. Thus, three genetic lineages of *R. helvetica* were revealed in samples from the Omsk region, and only one lineage was identified in samples from the Far East.

The observed high genetic heterogeneity of the *R. helvetica* population may be associated with a wide range of their vectors: *I. ricinus*, *I. pavlovsky*, *I. persulcatus*, *I. apronophorus*, *I. trianguliceps*, *Ixodes hexagonus* (Leach 1815), *Ixodes arboricola* (Schulze & Schlottke, 1929), *Ixodes ovatus* (Neumann, 1899) and *Ixodes monospinosus* (Saito, 1968) [7, 9, 12, 14, 32]. It should be noted that in *I. trianguliceps* only one sequence variant belonging to genetic lineage II was revealed, whereas in *I. apronophorus* five sequence variants belonging to three genetic lineages were revealed (Table 3). Sequences belonging to three lineages were identified in *I. persulcatus* ticks: lineage I in ticks from Western Siberia, lineage II in ticks from the Komi Republic, and lineage IV in ticks from the Far East. Such inconsistency may be associated with the significantly higher genetic variability of *I. apronophorus* and *I. persulcatus* ticks compared to *I. trianguliceps* [20].

At present, there is no reliable data to support the presence of co-feeding transmission of rickettsiae in ticks. Although such transmission can occur under artificial conditions (in the case of *Rickettsia rickettsia* (Wolbach 1919) Brumpt 1922)), its impact on pathogen transmission in nature appears to be negligible [33]. Our study of larvae collected from rodents revealed that there was no effective contained *R. helvetica* DNA transmission of *R. helvetica* between different species of *Ixodes* spp. during simultaneous feeding on small mammals. Actually, all *I. persulcatus* larvae collected from rodents were not infected with *R. helvetica*, *I. apronophorus* larvae contained *R. helvetica* DNA only from lineage III, and *I. trianguliceps* larvae only from lineage II (Tables 2, 3). Notably, the association between tick species and *R. helvetica* variants was observed even when larvae of different species were fed on the same rodent.

It should be noted that the data on genetic variability of *R. helvetica* are limited to a small number of studied regions and include mainly samples from Germany, the Komi Republic, the Omsk and Novosibirsk Regions, and the Far East. Further genotyping of *R. helvetica* specimens from other regions and different tick species is needed to assess the prevalence of different genetic lineages of this species. It is possible that different genetic lineages of *R. helvetica* may differ in their pathogenic properties.

CONCLUSION

In conclusion, a high level of infection of *I. apronophorus* ticks with the pathogenic for humans *R. helvetica* was revealed for the first time. High genetic variability was observed for *R. helvetica* samples from the Omsk region. For the first time, it was revealed that *R. helvetica* isolates could be reliably assigned to four genetic lineages, but no strict association of different *R. helvetica* lineages with a particular tick species or territory was observed.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Vera A. Rar – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: rarv@niboch.nsc.ru, https://orcid.org/0000-0002-5930-5306

Yana P. Igolkina – Cand. Sc. (Biol.), Junior Research Officer at the Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: igolkina@inbox.ru, https://orcid.org/0000-0001-5604-1846

Valeriy V. Yakimenko – Dr. Sc. (Biol.), Chief Research Officer, Head of the Laboratory of Arbovirus Infections of the Department of Natural Focal Viral Infections, Omsk Research Institute of Natural Focal Infections, e-mail: yakimenko_vv@oniipi.org, https://orcid.org/0000-0001-9088-3668

Artem Yu. Tikunov – Cand. Sc. (Biol.), Senior Research Officer, Head of the Laboratory of Antimicrobials, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: arttik@ngs.ru, https://orcid.org/0000-0001-5613-5447

Aleksey Yu. Nikitin – Dr. Sc. (Biol.), Leading Research Officer at the Zoological and Parasitological Department, Irkutsk Antiplague Research Institute of Siberia and Far East of Rospotrebnadzor, e-mail: nikitin_irk@mail.ru, https://orcid.org/0000-0002-3918-7832

Tamara I. Epikhina – Lead Engineer at the Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: tiepikhina@gmail.com, https://orcid.org/0009-0005-7077-1809

Nina V. Tikunova – Dr. Sc. (Biol.), Chief Research Officer, Head of the Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: tikunova@niboch.nsc.ru, https://orcid.org/0000-0002-1687-8278

IDENTIFICATION AND ANALYSIS OF CRISPR/CAS SYSTEMS STRUCTURES IN THE GENOMES OF ANTIBIOTIC-RESISTANT STRAINS OF *KLEBSIELLA PNEUMONIAE*

Stepanenko L.A. [1],
Sukhov B.G. [2],
Kon'kova T.V. [2],
Bedinskaya V.V. [1],
Klushina N.V. [2],
Zlobin V.I. [1]

[1] Irkutsk State Medical University (Krasnogo Vosstaniya str. 1, Irkutsk 664003, Russian Federation)
[2] Voevodsky Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences (Institutskaya str. 3, Novosibirsk 630090, Russian Federation)

Corresponding author:
Lilia A. Stepanenko,
e-mail: steplia@mail.ru

ABSTRACT

Background. *Klebsiella pneumoniae belongs to a group of opportunistic bacteria that can form multiple resistance to antibiotics and transmit it to various types of bacteria through horizontal gene transfer. These studies examine the structural and functional diversity of CRISPR/Cas systems that protect bacteria from foreign DNA. Their analysis using the example of antibiotic-resistant strains of Klebsiella pneumoniae will demonstrate their resistance to certain bacteriophages, which will make it possible to develop approaches to the treatment of complex infectious diseases caused by these microorganisms by creating targeted phage therapy.*

The aim of the study. *To perform a bioinformatics analysis of the identified structural components of CRISPR/Cas systems for screening bacteriophages through CRISPR cassette spacers using the example of antibiotic-resistant strains of Klebsiella pneumoniae.*

Materials and methods. *The article analyzed 29 full-genome sequences of Klebsiella pneumoniae, in the genome of which the structures of CRISPR/Cas systems and antibiotic resistance genes were determined (according to NCBI). To achieve this goal, using software modeling methods, a search was made for Cas genes and CRISPR cassettes, and their structural and functional characteristics were given.*

Results. *Using bioinformatic search algorithms in the genome of antibiotic-resistant strains, functionally active CRISPR/Cas systems with the presence of one or two CRISPR cassettes and belonging to Type I Subtype IE were identified. Groups of resistant strains with identical spacer composition of CRISPR cassettes have been identified. A phylogenetic analysis was carried out confirming their common origin. By analyzing the spacer sequences of CRISPR cassettes, the spectrum of diversity of phages of bacteria of the genus Klebsiella, Salmonella, belonging to the same family Enterobacteriaceae, was determined. Thus, information was obtained about the bacteriophages that are targeted by the action of CRISPR systems of Klebsiella pneumoniae strains that have antibiotic resistance.*

Conclusions. *Analysis of the functional and structural features of the CRISPR/Cas systems of antibiotic resistant Klebsiella pneumoniae strains made it possible to obtain information about their evolutionary history and about the bacteriophages against which their action is directed, that is, about their phage resistance. The approach used in this study may serve as the basis for the creation of personalized phage therapy.*

Key words: *Klebsiella pneumoniae, spacer, antibiotic resistance, CRISPR/Cas-system, bacteriophage, protospacer, bioinformatics*

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ИДЕНТИФИКАЦИЯ И АНАЛИЗ СТРУКТУР CRISPR/CAS-СИСТЕМ В ГЕНОМАХ АНТИБИОТИКОРЕЗИСТЕНТНЫХ ШТАММОВ *KLEBSIELLA PNEUMONIAE*

Степаненко Л.А. [1],
Сухов Б.Г. [2],
Конькова Т.В. [2],
Бединская В.В. [1],
Клушина Н.В. [2],
Злобин В.И. [1]

[1] ФГБОУ ВО «Иркутский государственный медицинский университет» Минздрава России (664003, г. Иркутск, ул. Красного Восстания, 1, Россия)
[2] ФГБУН Институт химической кинетики и горения им. В.В. Воеводского СО РАН (630090, г. Новосибирск, ул. Институтская, 3, Россия)

Автор, ответственный за переписку:
Степаненко Лилия Александровна,
e-mail: steplia@mail.ru

РЕЗЮМЕ

Обоснование. *Klebsiella pneumoniae относится к группе бактерий-оппортунистов, обладающих способностью формировать множественную антибиотикорезистентность и передавать её разным видам бактерий путём горизонтального переноса генов. Данные исследования посвящены изучению структурного и функционального разнообразия CRISPR/Cas-систем, защищающих бактерии от инородной ДНК. Их анализ на примере антибиотикорезистентных штаммов Klebsiella pneumoniae продемонстрирует их устойчивость к определённым бактериофагам, что позволит разработать подходы в лечении сложных инфекционных заболеваний, вызванных данными микроорганизмами, путём создания таргетной фаговой терапии.*

Цель исследований. *Выполнить биоинформатический анализ выявленных структурных компонентов CRISPR/Cas-систем для скрининга отбора бактериофагов через спейсеры CRISPR-кассет на примере антибиотикорезистентных штаммов Klebsiella pneumoniae.*

Материалы и методы. *В статье проанализированы 29 полногеномных последовательностей Klebsiella pneumoniae, в геноме которых были определены структуры CRISPR/Cas-систем и гены антибиотикорезистентности (по данным NCBI). Для решения поставленной цели с помощью программных методов моделирования произведён поиск Cas-генов и CRISPR-кассет, дана их структурная и функциональная характеристики.*

Результаты. *При помощи биоинформационных алгоритмов поиска в геноме антибиотикорезистентных штаммов были определены функционально активные CRISPR/Cas-системы с наличием одной или двух CRISPR-кассет и относящиеся к Type I Subtype IE. Определены группы резистентных штаммов, обладающие идентичным спейсерным составом CRISPR-кассет. Проведён филогенетический анализ, подтверждающий их единое происхождение. Путём анализа спейсерных последовательностей CRISPR-кассет определён спектр разнообразия фагов бактерий рода Klebsiella, Salmonella, относящихся к одному семейству Enterobacteriaceae. Таким образом, была получена информация о бактериофагах, на которые нацелено действие CRISPR-систем штаммов Klebsiella pneumoniae, обладающих антибиотикорезистентностью.*

Заключение. *Анализ функциональных и структурных особенностей CRISPR/Cas-систем антибиотикорезистентных штаммов Klebsiella pneumoniae позволил получить информацию об их эволюционной истории и о бактериофагах, против которых направлено их действие, то есть об их фагоустойчивости. Использованный в данный исследованиях подход в дальнейшем может послужить основой для создания персонифицированной фаготерапии.*

Ключевые слова: *Klebsiella pneumoniae, антибиотикорезистентность, CRISPR/Cas-система, спейсер, протоспейсер, бактериофаг, биоинформатика*

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INTRODUCTION

Antibiotics are one of mankind's greatest achievements. With their invention, previously fatal diseases became curable. Nevertheless, the formation of microorganism resistance to antibacterial drugs is one of the most serious problems of the health care system today [1]. The prevalence of antibiotic-resistant strains is increasing worldwide [2–6]. Infectious diseases caused by these pathogens are characterized by a longer course, adherence to numerous complications, increased disability and mortality [7–9]. As a result, the economic costs associated with the increase in the duration and cost of treatment of patients in hospital significantly increased [10, 11]. In February 2017, the World Health Organisation (WHO) published a list of bacterial pathogens that require new treatment approaches as a consequence of their high virulence and antibiotic resistance. *Klebsiella pneumoniae* belongs to this group. It is a Gram-negative opportunistic pathogen causing various infectious diseases including urinary tract infections, bacteremia, pneumonia and abscesses [12, 13]. The worldwide spread of Klebsiella pneumoniae clones producing extended-spectrum beta-lactamases (ESBL) and *Klebsiella pneumoniae* carbapenemase (KPCs) has become a serious threat to healthcare facilities, as they are a major cause of life-threatening nosocomial infections [14]. The widespread prevalence of strains with multiple antibiotic resistance necessitates the search for alternative methods to combat them. WHO has initiated numerous campaigns to combat antibiotic resistance. Epidemiologic surveillance of microbial resistance to antimicrobial agents has been launched [1]. New approaches are being created in the treatment of infectious diseases that reduce or completely replace the use of antibiotics [15]. Against this background, there is a renewed interest in the use of bacteriophages for the treatment of diseases of bacterial origin in medical practice. One of the real tools of effective control is the recently discovered CRISPR/Cas system in bacteria, which protects bacteria from alien mobile genetic elements. Bacteria are capable of acquiring and integrating genetic elements into their own genome [16]. In this manner, they preserve the genetic record of previous attacks by foreign nucleic acids in CRISPR arrays. These arrays consist of short and conserved repetitive sequences called repeats, which are strategically placed between unique sequences called spacers. They are being integrated by specialised Cas proteins into the CRISPR array during infections by nucleic acid invasion [17–20]. Adaptive immunity of prokaryotes against foreign genetic elements is achieved through the formation of effector complexes of RNA-oriented endonucleases, which have the ability to detect and incise foreign DNA previously integrated into the CRISPR array upon secondary infection [17, 21]. Thus, the study of the peculiarities of the structure and functioning of CRISPR/Cas systems of Klebsiella pneumoniae strains with antibiotic resistance will provide an opportunity to work out approaches for the development of targeted phage therapy in the treatment of complex infectious diseases.

THE AIM OF THE STUDY

Perform bioinformatics analysis of the identified structural components of CRISPR/Cas systems for screening bacteriophages through CRISPR cassette spacers using antibiotic-resistant *Klebsiella pneumoniae* strains as an example.

MATERIALS AND METHODS

The study was performed at the Laboratory of Molecular Virology and Biotechnology of the Research Institute of Biomedical Technologies of Irkutsk State Medical University. The system design of the conducted research is presented in Figure 1.

In these studies, 50 full-genome sequences of *Klebsiella pneumoniae* were randomly identified in the genome of which CRISPR/Cas systems were revealed by a bioinformatic method. The object of the study was 29 out of 50 full genome sequences of *Klebsiella pneumoniae*, downloaded from GenBank database, in the genome of which antibiotic resistance genes were revealed (according to NCBI (National Center for Biotechnology Information)). To address this goal, the developed bioinformatics software algorithm was used to search for CaS genes and CRISPR cassettes, and their structural and functional characteristics were obtained.

Software modeling methods were used to search for CRISPR/Cas systems and *Cas* genes: Macromolecular System Finder (MacSyF, ver. 1.0.2) with the auxiliary packages makeblastDB (ver. 3.0) and HMMER (ver. 2.2.28). CRISPR cassettes in genomes were detected and analyzed using online services of available programs: CRISPRCasFinder [22] and CRISPROne [23]. To search for phages, the decoded spacer sequences in FASTA format were uploaded to the CRISPRTarget online application. Only those strains in whose genome CRISPR/Cas-systems were determined according to the results of all used programs were considered in this study.

Phylogenetic trees were constructed and aligned using the MEGA X program using the nearest neighbor joining method (NJ) with bootstrap topology statistical significance analysis (number of replicates – 500) and using the Maximum Composite Likelihood genetic distance model. To "root" the tree, a strain of another *Escherichia coli* species (NC 000913.3) was added to the sample of test organisms and constituted an outgroup.

RESULTS AND DISCUSSION

Twenty-nine out of 50 strains with CRISPR/Cas system present in the genome and possessing antibacterial drug resistance genes (according to NCBI) served as the object of the study. Of the 29 strains tested, 55.2 % ($n = 16$) had *Klebsiella pneumoniae* carbapenemase activity, 3.4 % ($n = 1$) each were resistant to rifampicin and trimethoprim-sulfamethoxazole and 11 (37.9 %) strains had multiple antibiotic resistance.

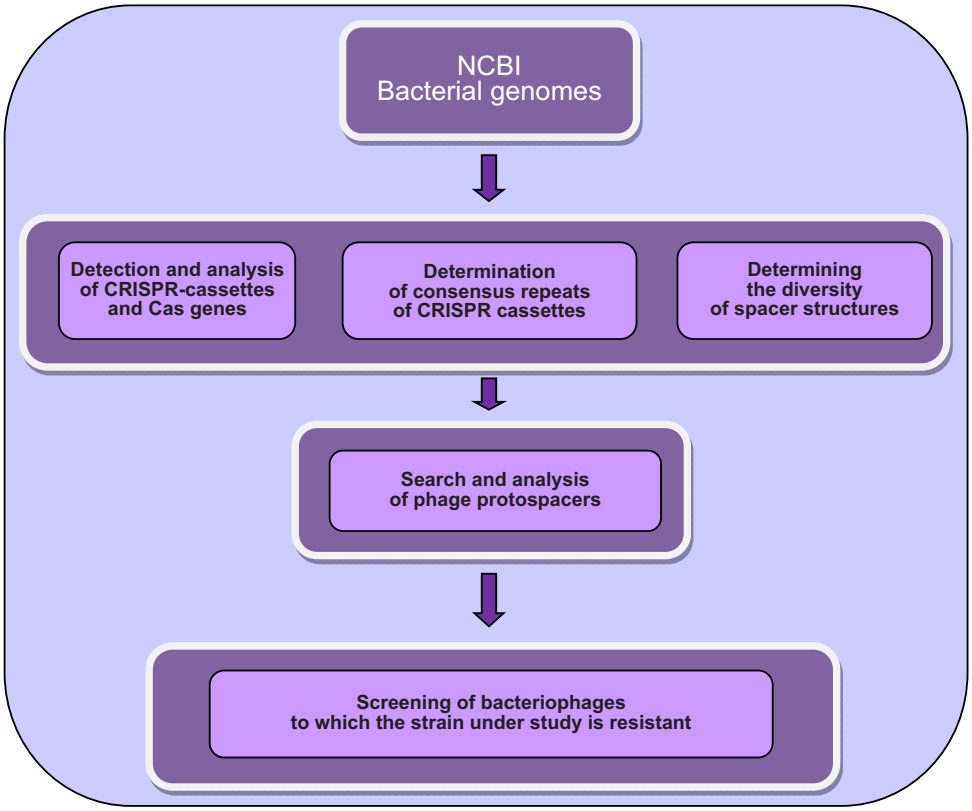

FIG. 1.
Scheme of systematic research design

FIG. 2.
Arrangement of CRISPR cassettes and Cas genes of the Type I Subtype IE system in the genome of Klebsiella pneumoniae strains (data from GenBank: NZ_CP011624.1, NZ_CP006798.1)

Using several bioinformatic search programs, the presence of one or two CRISPR cassettes was revealed in the CRISPR/Cas systems of the strains studied. In all cases, a complete set of *Cas* genes characteristic of Type I Subtype IE systems (*cas2*, *cas1*, *cas5*, *cas7*, *cas6*, *cse2gr11*, *cas8*, *cas3*) was revealed next to the cassettes, which indicates the functional activity of CRISPR/Cas systems of the studied bacteria (Fig. 2).

In all CRISPR cassettes examined, the number of revealed spacers was 1629. Among them, 498 spacers were non-repeatable, and 276 spacers had repeats at two or more CRISPR loci. No spacer repeats were recorded within the cassettes. The number of spacers in the cassettes ranged from 4 to 64. The total number of spacers was greater in strains containing one cassette compared to strains containing two cassettes.

Two types of consensus sequences of CRISPR cassette repeats were observed during the study. This may indicate the presence of a certain type of CRISPR system (in our case it is Type I Subtype IE) and its rather stable operation, since these repeats are recognized by Cas endonuclease proteins, the action of which is aimed at cutting and destruction of the target (Fig. 3).

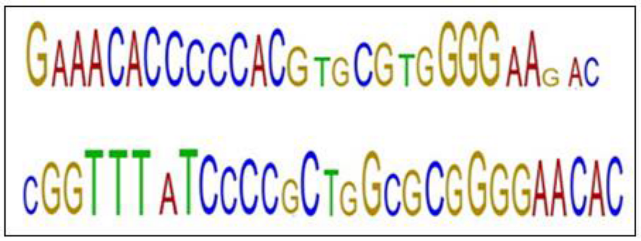

FIG. 3.

Nucleotide sequence of CRISPR consensus repeats of antibiotic-resistant Klebsiella pneumoniae strains

Screening of CRISPR cassette spacer sequences of the strains under study showed their complete correspondence to the protospacers of phages of bacteria of the family *Enterobacteriaceae* of the genus *Klebsiella* (Table 1). It is important to note that for most spacers, there was no complete correspondence between the protospacers of phages from known databases.

In the analysis it was found that in CRISPR cassettes of the studied strains (NZ_CP013322.1) there was a correspondence of the site of one spacer (6) to protospacers of several phages of bacteria of the same family, and correspondence of several spacers (52, 55) of one strain (NZ_CP017934.1) to protospacers of the same phage. This may indicate that during evolution, bacteria have adapted to acquire only those phage DNA spacers that are efficiently recognized by their effector complex, thus enhancing the protective effect of CRISPR/Cas systems.

Therefore, the full spectrum of bacteriophages detected through spacer sequences of CRISPR cassettes of antibiotic-resistant *Klebsiella pneumoniae* strains with access to their complete genome in GenBank was determined. Considering the principle of operation of bacteria CRISPR/Cas-systems, it can be assumed that when encountering these phages, they will be destroyed by Cas-nucleases provided that a part of its genome corresponds to the sequence of the spacer in the CRISPR-cassette of bacteria. This approach can be used in the future as a basis for the creation of personalized phagotherapy.

Next, groups of bacteria possessing identical CRISPR spacer cassettes were revealed by analyzing antibiotic-resistant strains. The first group consisted of two strains possessing a different combination of *Klebsiella pneumoniae* carbapenemase genes (NDM-1, OXA-48, OXA-181) that were associated with other resistance determinants, including extended-spectrum β-lactamases and ArmA methylase encoding resistance to aminoglycosides. So, they were defined as pan-resistant, as they possessed multiple antibiotic resistance. Among the antibiotic-resistant bacteria, five strains were revealed. It should be noted that these strains, having similar spacer composition, were isolated in different regions of the world (South Korea, China (Shanghai)) and at different times (2013, 2014) (Table 2).

The second group consisted of three strains with multiple antibiotic resistance isolated in the same hospital (Greece) but at different times (2011, 2013). They were identified as intrahospital strains. One CRISPR cassette was identified in each of their genomes. Furthermore, two strains had the same spacer cassette composition represented by 24 spacers (Table 3).

The third group consisted of eight *Klebsiella pneumoniae* carbapenemase-producing strains isolated from patients treated at a hospital in Lower Saxony, Germany, where a nosocomial outbreak caused by *Klebsialla pneumoniae* was reported. These strains were found to have one CRISPR cassette each in the genome, consisting of 35 spacers that were completely identical to each other. Also included in this group were three other strains with similar spacer cassette composition but isolated in different regions of the world (Arab Emirates, USA, Singapore) and at different times (2015, 2016, 2014).

The similarity of the spacer composition of cassettes in the genomes of the strains of the represented groups may indicate their common origin. It can be assumed that as a result of widespread circulation, strains genetically changed but retained the CRISPR cassette structure, while in hospital conditions (in-hospital outbreaks) they exchanged genetic information, acquiring new properties but also retaining the CRISPR cassette structure. To confirm this point, a phylogenetic analysis of the strains under study was performed based on the 16S rRNA nucleotide sequence (Fig. 4).

Phylogenetic analysis revealed that strains with similar spacer composition of CRISPR cassettes in the studied groups are closely related, as they are located on branches having the same ancestor (one node). At the same time, the strain (NZ_CP012745.1), which is a member of group 2 and has an individual spacer composition of CRISPR cassettes, has no common ancestor with the other representatives of this group.

TABLE 1

DIVERSITY OF SPACERS AND THEIR CORRESPONDING PROTOSPACER PHAGES IN THE GENOMES OF ANTIBIOTIC-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

Strain	Cassette/spacer	Bacteriophage	GenBank access number
NZ_CP008929.1	1/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP009208.1	1/2	*Klebsiella* phage ST512-KPC3phi13.1	MK448235
NZ_CP009208.1	1/2	*Klebsiella* phage ST11-VIM1phi8.1	MK448233
NZ_CP009208.1	2/4	*Klebsiella* phage ST147-VIM1phi7.2	MK448232
NZ_CP009208.1	2/4	*Klebsiella* phage 1 LV-2017	KY271401
NZ_CP012426.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP012743.1	1/12	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP012744.1	1/12	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP012745.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP012745.1	1/22	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925
NZ_CP012745.1	1/25	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP013322.1	1/6	*Klebsiella* phage ST512-KPC3phi13.1	MK448235
NZ_CP013322.1	1/6	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP013322.1	1/6	*Klebsiella* phage ST11-VIM1phi8.1	MK448233
NZ_CP013322.1	1/6	*Klebsiella* phage 2 LV-2017	KY271396
NZ_CP013322.1	1/6	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP015382.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP015500.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP015500.1	1/22	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925
NZ_CP015500.1	1/25	*Klebsiella* phage 2b LV-2017	KY271395
NZ_FO834906.1	1/10	*Klebsiella* phage ST11-VIM1phi8.2	MK448234
NZ_CP022127.1	2/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP017934.1	1/53	*Klebsiella* phage ST405-OXA48phi1.2	MK416007
	1/55	*Klebsiella* phage 1 LV-2017	KY271401
	1/22	*Klebsiella* phage ST101-KPC2phi6.3	MK416017
	1/36	*Klebsiella* phage 2b LV-2017	KY271395
	1/52	*Klebsiella* phage ST101-KPC2phi6.3	MK416017
	1/52	*Klebsiella* phage ST147-VIM1phi7.2	MK448232
	1/52	*Klebsiella* phage 1 LV-2017	KY271401
	1/18	*Klebsiella* phage ST16-OXA48phi5.1	MK416013
	1/18	*Klebsiella* phage 5 LV-2017	KY271399
NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/3	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/14	*Klebsiella* phage YMC1601N133_KPN_BP	MF476925

TABLE 1 (*continued*)

NZ_CP018140.1 NZ_CP018686.1 NZ_CP018695.1 NZ_CP018701.1 NZ_CP018707.1 NZ_CP018713.1 NZ_CP018719 NZ_CP017985.1	1/17	*Klebsiella* phage 2b LV-2017	KY271395
NZ_CP018458.1	2/6	*Klebsiella* phage ST13-OXA48phi12.4	MK422450
NZ_CP019047.1	2/11	*Klebsiella* phage 2 LV-2017	KY271396
NZ_CP019047.1	2/5	*Klebsiella* phage ST11-OXA245phi3.2	MK416010
NZ_CP019047.1	2/13	*Klebsiella* phage ST101-KPC2phi6.1	MK448231
NZ_CP019047.1	2/12	*Klebsiella* phage ST16-OXA48phi5.2	MK448230

TABLE 2

SPACER COMPOSITION OF CRISPR CASSETTES OF THE FIRST GROUP OF PAN-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

No. of strain in GenBank	NZ_CP014004.1	NZ_CP012753.1
Place and year of strain isolation	China, Jiangxi 2014	South Korea 2013
Cassette 1	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG
	CGTGTCGAAGCGCACCTCGTAGCCGAGCCAGTC	CGTGTCGAAGCGCACCTCGTAGCCGAGCCAGTC
	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC
	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA
	TATCGTGCAGAGTCACAACCTGACGGGATTATC	TATCGTGCAGAGTCACAACCTGACGGGATTATC
	TCGTGCATGGTGAGGATTCTACAGTCGCACCAT	TCGTGCATGGTGAGGATTCTACAGTCGCACCAT
	TACCTCCCGGCGTCCGCGCCAGGGCGATCACGTG	TACCTCCCGGCGTCCGCGCCAGGGCGATCACGTG
	CCTGCAGCTGGCCGTCGAGCTGACGGATGCCGG	CCTGCAGCTGGCCGTCGAGCTGACGGATGCCGG
	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT
	TATCATCCCTATCGCGCAGCACTTCGACGGCGA	TATCATCCCTATCGCGCAGCACTTCGACGGCGA
	TACCGCCGCGATACTGGCAGTTTTCAGCTGAAT	TACCGCCGCGATACTGGCAGTTTTCAGCTGAAT
	TCCCGCTGGGTAAGCAATATATAACATTTGCAG	
	TTTAACATTCTGAAAGTGCAATTTTTGAGGCTC	
	TTAAGGAGGGGCGCCATGGAGCCCGTATTGATT	
Cassette 2	CAGCGGCGGCGGTAACGCCGCCAGGAGCAACCT	CAGCGGCGGCGGTAACGCCGCCAGGAGCAACCT
	CACCGATCTGCGCCAGCTGGGTGAGACGATGAC	CACCGATCTGCGCCAGCTGGGTGAGACGATGAC
	TACACTCAAGAAAACAAAATCTCAGGTTGATAC	TACACTCAAGAAAACAAAATCTCAGGTTGATAC
	TGGAAGGCGCGATTTGGAGATAGAGCAGCATGA	TGGAAGGCGCGATTTGGAGATAGAGCAGCATGA
	CTCGCACAGCATCGCCCGGATCCGCTTCCACGC	CTCGCACAGCATCGCCCGGATCCGCTTCCACGC
	CACCCGCGTTTTCGAAAGGGATGGCGGCTATGT	CACCCGCGTTTTCGAAAGGGATGGCGGCTATGT
	CGACGGGGCAGGTTTACGTCTACCCGGGCAGGG	CGACGGGGCAGGTTTACGTCTACCCGGGCAGGG
	CATACCAGTCTCCGCCGCGGTCGTACTCAATAT	CATACCAGTCTCCGCCGCGGTCGTACTCAATAT
	TCCGCCGTTTAATCGCGGTGATGATATCCGGCA	TCCGCCGTTTAATCGCGGTGATGATATCCGGCA
	GGAATCCACGACGCGCCGTACCAGCGCGGGCATTCGTTCT	TGGAATCCACGACGCGCCGTACCAGCGCGGGCATTCGTTCT

Note. Identical spacers are highlighted in color.

TABLE 3

SPACER COMPOSITION OF CRISPR CASSETTES OF THE SECOND GROUP OF RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS

No. of strain in GenBank	NZ_CP012743.1	NZ_CP012744.1	NZ_CP012745.1
Place and year of strain isolation	Greece 2011	Greece 2013	Greece 2013
Cassette 1	CATTTCATAGTGATTCGACTATTTAATTAACA	CATTTCATAGTGATTCGACTATTTAATTAACA	GAGCAGGCACCCGCCGCAACGACGAAGAGCGC
	AGTTCACGACAGGCAAAGTCTTTACGGTATGC	AGTTCACGACAGGCAAAGTCTTTACGGTATGC	AAATCAGCCAGCACCACGATTCTGGGAAATTT
	TCTGCTGGTTACAGGAGAAAAAAATGATTGGT	TCTGCTGGTTACAGGAGAAAAAAATGATTGGT	ACAGGCTTACCCGTATTGAGACGGTTGCTGAA
	TTATTTCTAAACTAAGTTTTGTTTCATGCAGT	TTATTTCTAAACTAAGTTTTGTTTCATGCAGT	GAAACCCCATCAGATGACCCTCCCCATGTTGGC
	TTTTTTTGACGAAGACGGCAACGAGTTAGAAG	TTTTTTTGACGAAGACGGCAACGAGTTAGAAG	TTGCCTGGTCTGCTTGGTGATGATCCGTGGTA
	GTCTCTGCCAGTTTTACCTGCTCAGCGGATAA	GTCTCTGCCAGTTTTACCTGCTCAGCGGATAA	TACAGAACGACTGAGGCGGCGTGTATTGCATA
	CTGACAGCTGGCGCTAACCCGTCGTTTATCGC	CTGACAGCTGGCGCTAACCCGTCGTTTATCGC	GATCTTAACTCTATTGCCAATGGCGCAATTCA
	CGTGAGCATCTGGGCATCTTCAGTGATAGCGT	CGTGAGCATCTGGGCATCTTCAGTGATAGCGT	GGCGATGCGCGCTCTGCTGGCTATCGGTAAAA
	TATTTTTGAGATGATGGATTGTTGCACACCAG	TATTTTTGAGATGATGGATTGTTGCACACCAG	AATGCAGCAACCGGCAAATATATCGCCGGTAA
	CGCTTCTCGGCTTCTCTGAATTTATCCGCCCA	CGCTTCTCGGCTTCTCTGAATTTATCCGCCCA	GGGCTGCCGCACGCCTGGGACGAGTCGAGCCC
	ATCCCGACCCGCTCCTCCAGAGCGAATACGAT	ATCCCGACCCGCTCCTCCAGAGCGAATACGAT	CCGCAATAACAAAAATAAATGAGGGTTAAAGT
	TGCTTTATGGCAAATAAGAGAGGATATAACCA	TGCTTTATGGCAAATAAGAGAGGATATAACCA	GTAAATGGGAATGAGTAGAAGAGCGTCATTGG
	GCGTCCGCCTCCCAAATAGCCAGGCTGTTATT	GCGTCCGCCTCCCAAATAGCCAGGCTGTTATT	CCCCCGCGCACATGCTTAAACGCGCTATCACG
	TAAATCCCAGCTGGTTTGACGTAGTGGCACTG	TAAATCCCAGCTGGTTTGACGTAGTGGCACTG	GGCATCTGTTGTGTAATGTTGAGTTTTTTTCA
	CAATGAAGGCTTATAAGGGTGAAGAGAGCAGG	CAATGAAGGCTTATAAGGGTGAAGAGAGCAGG	CAGGTTAAACATGTAAAAAATGACCGTCGCCG
	CAGTCGGTAACGGCTGGGCGTGACCTCAAAGC	CAGTCGGTAACGGCTGGGCGTGACCTCAAAGC	CACATTGCCCGGTCTGAAAAGTATTTGAAAAT
	GTGCCATTTTTATTTTGCTTAAAATAAATATC	GTGCCATTTTTATTTTGCTTAAAATAAATATC	TCCGCACAGTCAAACGCTCCAGACACCAACCC
	CCTATTGTGTTCAGTCGTAACAGCTGAATCAG	CCTATTGTGTTCAGTCGTAACAGCTGAATCAG	CCGGAACACCACCAGTAACAGCTACTGTAGGC
	GGCGCGGCGGAATAACCACTTTATGAGCAGGT	GGCGCGGCGGAATAACCACTTTATGAGCAGGT	TGACCCTGTTGATTTTGTTCCAGGTAATACGT
	GCGACCATGCCGTAGTCTTCAATGACGTAATC	GCGACCATGCCGTAGTCTTCAATGACGTAATC	TTAACCTCGTCGTTCTGGTTTCCGCCCAGGAT
	CCCCGTCGTCATTCGCGCATTCTGCGCACAGA	CCCCGTCGTCATTCGCGCATTCTGCGCACAGA	GAACCTGAATTCGAAGGGTGGGTCATCCTTCC
	TAACAGGGCCATTTTCCCGGGTTGCCAGACGA	TAACAGGGCCATTTTCCCGGGTTGCCAGACGA	GGACCCCGAGCGACCCGGTCACCCTCCGACCT
	TCACCCTCGTAGCCGATACCACTTTCGCGCAG	TCACCCTCGTAGCCGATACCACTTTCGCGCAG	CCGTCGAACGGCGGTTATATCCATCTTGAGTC
	GGGTTCACTTGGGTGAAACTGAACTAACT	GGGTTCACTTGGGTGAAACTGAACTAACT	ACCGATCCCACAATTGCGGCGGTTGAGATTGA

The detection of similar spacers in CRISPR cassettes of different strains may thus indicate phylogenetic relationships between strains. This technique can be used in epidemiologic analysis of outbreaks caused by antibiotic-resistant strains of *Klebsiella pneumoniae*.

Screening of bacteriophages by spacer sequences of CRISPR cassettes of strains from the above groups is presented in Table 1, with the exception of two pan-resistant strains from the first group (NZ_CP014004.1 and NZ_CP012753.1), as no complete correspondence of spacers in the composition of their cassettes to protospacers from known databases was revealed. The analysis of the spacer sequences, however, resulted in the highest correspondence to the protospacers of phages of *Klebsiella* and *Salmonella* bacteria belonging to the same family Enterobacteriaceae (Table 4).

Of significance, the CRISPR cassettes of the strains studied revealed the correspondence of one of the spa-

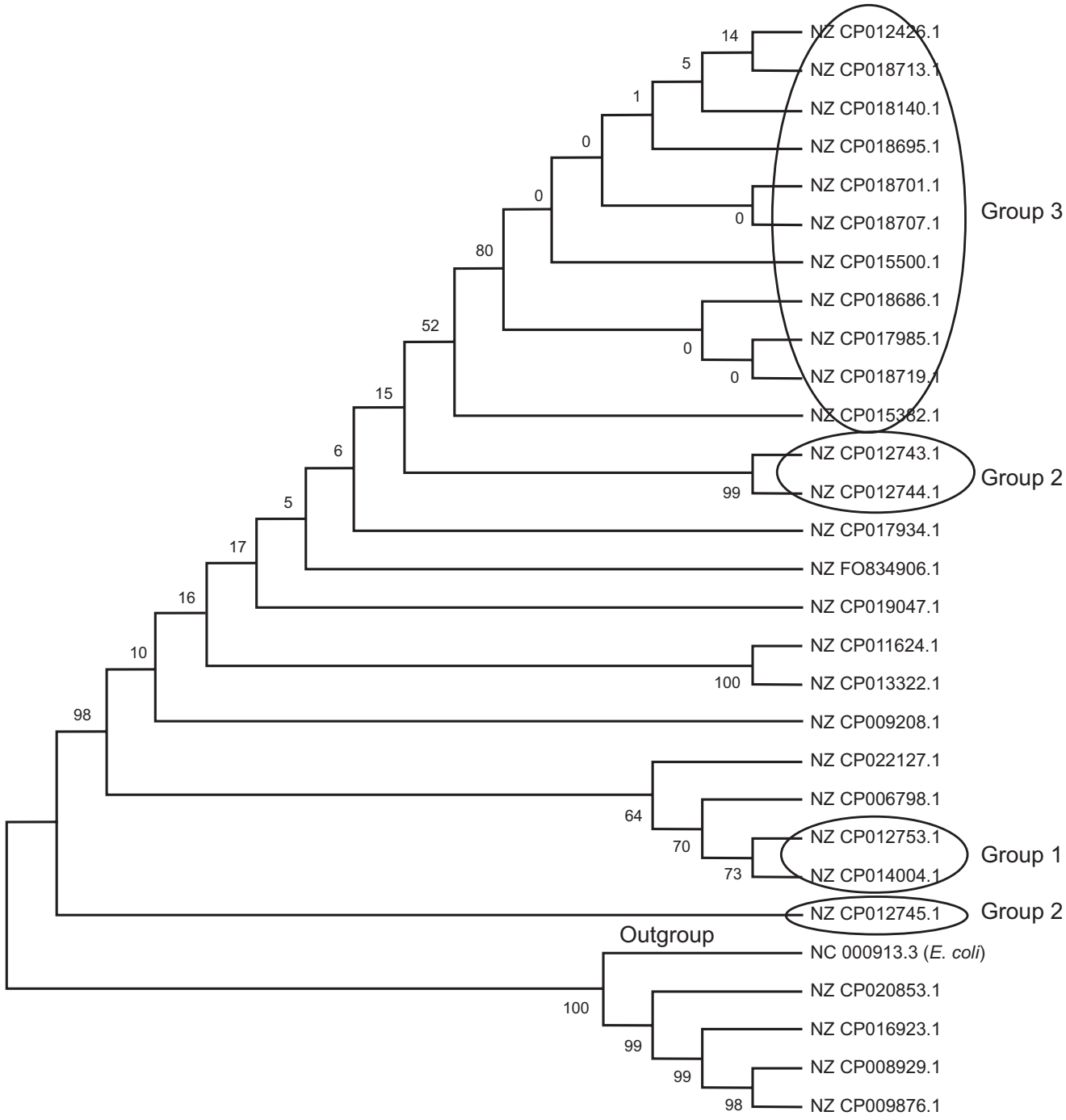

FIG. 4.

Phylogenetic tree based on the 16S rRNA marker of antibiotic-resistant Klebsiella pneumoniae strains (Maximum Composite Likelihood model of genetic distances was used)

cers to protospacers of several bacterial phages belonging to the same family. This may indicate the conservativity of the acquired new spacers from phage DNA regions. In this way, the bacterium "one spacer" protects itself against multiple phages. The presence of this mechanism increases the efficiency of the protective action of CRISPR/Cas systems.

The study of the identified bacteriophages revealed that all *Klebsiella* phage are prophages of *Klebsiella pneumoniae* bacteria belonging to isolates which are part of clonal group ST 307 (according to NCBI data) producing *Klebsiella pneumoniae* carbapenemase (Table 5). The ST 307 genome has been demonstrated to encode genetic features that may provide an advantage

TABLE 4

DIVERSITY OF BACTERIOPHAGES CORRESPONDING TO IDENTICAL SPACERS IN THE FIRST GROUP OF *KLEBSIELLA PNEUMONIAE* STRAINS WITH MULTIPLE ANTIBIOTIC RESISTANCE

GenBank strain access number	Nucleotide sequence of the spacer	Bacteriophage	GenBank bacteriophage access number	Number of nucleotide substitutions
NZ_CP014004.1 NZ_CP012753.1	TTCATCACGTGTGAGCGGATTTGGCTCTATCCT	*Klebsiella* phage 6 LV-2017	KY271400	2
NZ_CP014004.1 NZ_CP012753.1	TGCCTCCAATGCAATCACCGGCCTGCTAACCGG	*Klebsiella* phage ST101-KPC2phi6.1	MK448231	1
NZ_CP014004.1 NZ_CP012753.1	CGTCATCAGCGCCTTGTTCCAGCGGCGACCACC	*Salmonella* phage FSL SP-062	KC139634	2
		Klebsiella phage ST512-KPC3phi13.1	MK448235	1
		Klebsiella phage ST11-VIM1phi8.1	MK448233	1
NZ_CP014004.1 NZ_CP012753.1	TCCAGTCGTCGTAGTCCTCGGTAATGTCCTCGA	*Klebsiella* phage ST101-KPC2phi6.1	MK448231	1
		Klebsiella phage 2b LV-2017	KY271395	1
		Klebsiella phage 2 LV-2017	KY271396	2

TABLE 5

LIST OF IDENTIFIED BACTERIOPHAGES IN THE FIRST GROUP OF *KLEBSIELLA PNEUMONIAE* STRAINS WITH MULTIPLE ANTIBIOTIC RESISTANCE

Bacteriophage	Bacteriophage Access Number (GenBank)	Source of bacteriophage isolation	Location of bacteriophage isolation
Klebsiella phage 6 LV-2017	KY271400	*Klebsiella pneumoniae* ST307	Италия, Рим
Klebsiella phage ST101-KPC2phi6.1	MK448231	*Klebsiella pneumoniae* ST101-KPC2	Испания, Ла-Корунья
Salmonella phage FSL SP-062	KC139634	Молочная ферма	Нью-Йорк, США
Klebsiella phage ST512-KPC3phi13.1	MK448235	*Klebsiella pneumoniae* ST512-KPC3	Испания, Ла-Корунья
Klebsiella phage ST11-VIM1phi8.1	MK448233	*Klebsiella pneumoniae* ST11-VIM1	Испания, Ла-Корунья
Klebsiella phage ST101-KPC2phi6.1	MK448231	*Klebsiella pneumoniae* ST101-KPC2	Испания, Ла-Корунья
Klebsiella phage 2b LV-2017	KY271395	*Klebsiella pneumoniae* KH43 – клон *Klebsiella pneumoniae* ST307	Италия, Рим
Klebsiella phage 2b LV-2017	KY271396	*Klebsiella pneumoniae* H151440672 – клон *Klebsiella pneumoniae* ST307	Италия, Рим

in adaptation to the hospital environment and human host. All ST 307 isolates are encapsulated and therefore have a higher resistance to complement-mediated eradication. All this ensures the spread and prevalence of strains with this genetic sequence worldwide [24]. It is known that prophages remain latent in the genome for several cell divisions, but under the influence of external factors they change into virulent forms and lysate the bacterial cell. In summary, the antibiotic-resistant strains under study have a genetic memory of these bacteriophages in the form of spacers of their CRISPR cassettes.

This analysis of the strains under study showed a rather wide spacer composition of their CRISPR cassettes, diversity of bacteriophages detected through spacer sequences, which makes it possible to suggest that they possess not only antibiotic resistance, but also resistance to many bacteriophages. Consequently, a personalized approach to the integrated selection of antibiotics and bacteriophages will potentially help to address the treatment of diseases caused by these strains.

CONCLUSION

The results of these studies provided information about the structure of CRISPR/Cas systems in the genomes of antibiotic-resistant *Klebsiella pneumoniae* strains and their functional features. The revealed diversity of spacer sequences indicates the evolutionary history and adaptive capabilities of these strains. In this case, the detection of similar spacers in CRISPR cassettes of different strains may indicate phylogenetic relationships between strains. The study of phage-bacteria interaction patterns based on spacer and protospacer sequences makes it possible to determine the resistance and sensitivity of strains to specific bacteriophages. In this way, the putative resistance of antibiotic-resistant strains to specific phages was determined. The mechanism of studying CRISPR/Cas systems in bacterial genomes using bioinformatics analysis will in the future provide more complete information about the properties of causative agents, their evolution and the selection of highly specific bacteriophages.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Lilia A. Stepanenko – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Molecular Virology and Biotechnology, Research Institute of Biomedical Technologies, Irkutsk State Medical University, e-mail: steplia@mail.ru, https://orcid.org/0000-0002-5792-7283

Boris G. Sukhov – Cand. Sc. (Chem.), Leading Research Officer at the Laboratory of Nanoparticles, Voevodsky Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences, e-mail: boris_sukhov@mail.ru, https://orcid.org/0000-0002-9751-6454

Tatyana V. Kon'kova – Cand. Sc. (Chem.), Senior Research Officer at the Laboratory of Nanoparticles, Voevodsky Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences, e-mail: konbuivol_2@yahoo.com, https://orcid.org/0009-0002-0706-8692

Victoria V. Bedinskaya – Postgraduate at the Department of Microbiology, Virology and Immunology, Irkutsk State Medical University, e-mail: vika-2801@mail.ru, https://orcid.org/0009-0000-9536-4795

Nadezhda V. Klushina – Postgraduate, Leading Engineer at the Laboratory of Nanoparticles, Voevodsky Institute of Chemical Kinetics and Combustion, Siberian Branch of the Russian Academy of Sciences, e-mail: klusinanadezda677@gmail.com, https://orcid.org/0009-0008-7776-2931

Vladimir I. Zlobin – Dr. Sc. (Med.), Member of the RAS, Head of the Department of Microbiology, Virology and Immunology, Irkutsk State Medical University, e-mail: vizlobin@mail.ru, https://orcid.org/0000-0002-0164-5113

CORONAVIRUSES IN RODENTS AND INSECTIVORES IN ALTAI REPUBLIC

Yashina L.N. [1],
Abramov S.A. [2],
Smetannikova N.A. [1],
Malyshev B.S. [1],
Krivopalov A.V. [2],
Dupal T.A. [2]

[1] State Research Center of Virology and Biotechnology "Vector" (Koltsovo 630559, Novosibirsk Region, Russian Federation)
[2] Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences (Frunze str. 11, Novosibirsk 630091, Russian Federation)

Corresponding author:
Liudmila N. Yashina,
e-mail: yashina@vector.nsc.ru

ABSTRACT

Coronaviruses (family Coronaviridae, genera Alphacoronavirus, Betacoronavirus) are dangerous viral pathogens that have caused three outbreaks of severe respiratory diseases SARS, MERS, COVID-19. In Russia, data on coronaviruses in natural reservoirs are limited, as investigations began only during the COVID-19 pandemic.

The aim of the work. *To study the diversity of coronaviruses among rodents and insectivores in the Altai Republic.*

Materials and methods. *Rodents (n = 67) and shrews (n = 52) were captured in 2022. Samples were analyzed by reverse transcription-polymerase chain reaction followed by sequencing.*

Results and conclusions. *Four samples from rodents (Myodes rutilus, M. glareolus, Apodemus peninsulae, A. agrarius) and two samples from an insectivore (Crocidura sibirica) were positive for coronaviruses, among which three different coronaviruses were detected. Rodent-borne coronaviruses are classified in the genus Betacoronavirus, subgenera Embecovirus, and have shown host associated clustering. The nucleotide sequences of Siberian coronaviruses from rodents were identical for closely related species (M. rutilus and M. glareolus, A. agrarius and A. peninsulae) and close (> 94 % homology) to previously published sequences in each of the groups of carriers found in the territory Novosibirsk region, Europe and China. The coronavirus identified from the insectivore, possibly belonging to a new subgenera of the family Coronaviridae, has also been assigned to the genus Betacoronavirus.*

Conclusion. *Five species of natural carriers of three different coronaviruses were detected in the Altai Republic. A high level of identity of coronaviruses genomes from rodents has been revealed, indicating a relatively low rate of their evolution.*

Key words: *coronavirus, taxonomy, rodents, shrews, Siberia*

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КОРОНАВИРУСЫ, ЦИРКУЛИРУЮЩИЕ СРЕДИ ГРЫЗУНОВ И НАСЕКОМОЯДНЫХ В РЕСПУБЛИКЕ АЛТАЙ

Яшина Л.Н. [1],
Абрамов С.А. [2],
Сметанникова Н.А. [1],
Малышев Б.С. [1],
Кривопалов А.В. [2],
Дупал Т.А. [2]

[1] ФБУН «Государственный научный центр вирусологии и биотехнологии «Вектор» Роспотребнадзора (630559, р. п. Кольцово, Новосибирская область, Россия)

[2] ФГБУН Институт систематики и экологии животных СО РАН (630091, г. Новосибирск, ул. Фрунзе, 11, Россия)

Автор, ответственный за переписку:
Яшина Людмила Николаевна,
e-mail: yashina@vector.nsc.ru

РЕЗЮМЕ

Коронавирусы (семейство Coronaviridae, роды Alphacoronavirus и Betacoronavirus) относятся к числу опасных вирусных патогенов, вызвавших три вспышки тяжёлых респираторных заболеваний SARS, MERS, COVID-19. В России данные о коронавирусах, циркулирующих в природных резервуарах, ограничены, так как исследования начались только во время пандемии COVID-19.

Цель работы. *Исследование многообразия коронавирусов среди грызунов и насекомоядных в Республике Алтай.*

Материалы и методы. *Грызуны (67 особей) и насекомоядные (52 особи) были отловлены в 2022 г. Образцы проанализированы методом обратной транскрипции – полимеразной цепной реакции и последующим секвенированием.*

Результаты и обсуждение. *Положительными на коронавирусы оказались 4 образца от грызунов (Myodes rutilus, M. glareolus, Apodemus peninsulae, A. agrarius) и 2 образца от насекомоядного (Crocidura sibirica), в которых обнаружены 3 различных коронавируса. Ассоциированные с грызунами коронавирусы отнесены к роду Betacoronavirus, подроду Embecovirus и демонстрировали филогенетическое группирование в соответствии с видом природного носителя. Нуклеотидные последовательности сибирских изолятов коронавирусов от грызунов были идентичными для носителей близкородственных видов (M. rutilus и M. glareolus, A. agrarius и A. peninsulae) и близки (> 94 % гомологии) к ранее опубликованным последовательностям в каждой из групп носителей, обнаруженных на территории Новосибирской области, Европы и Китая. К роду Betacoronavirus отнесён и коронавирус, выявленный от насекомоядного, возможно, относящийся к новому подроду семейства Coronaviridae.*

Заключение. *Обнаружены 5 видов природных носителей 3 различных коронавирусов на территории Республики Алтай. Выявлен высокий уровень идентичности геномов коронавирусов от грызунов, свидетельствующий об относительно низкой скорости их эволюции.*

Ключевые слова: *коронавирус, таксономия, грызуны, насекомоядные, Сибирь*

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INTRODUCTION

Coronaviruses (family *Coronaviridae*, genera *Alphacoronavirus* and *Betacoronavirus*) are among the dangerous viral pathogens that have caused three outbreaks of severe respiratory diseases SARS, MERS, COVID-19 in the last two decades [1–3]. After the SARS outbreak in 2002–2003, epidemiological and etiological studies confirmed that SARS-CoV entered humans via civets [4]. Subsequent field and experimental studies showed that civet was only an intermediate host of SARS-CoV and bats were its natural host [5]. MERS-CoV, which first appeared in the Middle East in 2012, also originates from bats and infects humans via camels [6]. After COVID-19 was first revealed in Wuhan, its etiological agent, a novel coronavirus later named SARS-CoV-2, was not only immediately identified based on genome studies, but also the virus was found to have high homology with a known bat coronavirus identified in 2018 [3, 7, 8]. Additionally, recent studies have revealed that human coronaviruses such as OC43, 229E, HKU1 and NL63 also originate from bats or rodents [9, 10].

Coronaviruses are characterized by high genetic diversity in wild small mammals. Over a long evolutionary history, viruses have not only co-evolved with their hosts, but have also often crossed the interspecies barrier and spread between species and evolved to adapt to new hosts [11]. A very diverse range of coronaviruses has been consistently detected in rodents in recent years, some of which circulate simultaneously in several rodent species or even several subfamilies [12–15]. Recent studies have shown that OC43 and HKU1 coronaviruses, which cause human respiratory diseases, can also be originated from rodents [9, 16]. Along with rodents, small mammals such as shrews, which are also highly genetically diverse, can also be carriers of various coronaviruses. Shrew coronaviruses have recently been discovered to have important evolutionary significance [17].

The problem of coronavirus research is relevant for Russia, since no epidemiological studies were conducted to reveal coronaviruses in natural reservoirs before the pandemic. Only in 2020, SARS-like coronaviruses (Khosta-1 and Khosta-2) were identified in two species of bats of the horseshoe bat genus in the Krasnodar region of the Russian Federation and their full-length genomes were studied [18]. Subsequent studies revealed that the receptor-binding domain of one of the novel coronaviruses is capable of utilising the human ACE2 receptor and poses a potential risk to humans [19]. Another coronavirus was revealed among Pipistrellus bats in the central part of European Russia [20]. The evolutionary history of this novel MERS-like coronavirus suggests recombination of the ancestral genomes from bat and hedgehog. Studies of coronaviruses among rodents and insectivores were first conducted in Siberia (Novosibirsk Region) [21]. Circulation of viral coronaviruses was found in three species of rodents and one species of insectivores. The rodent-associated coronaviruses belonged to the *Betacoronavirus* genus, evidenced phylogenetic grouping according to the natural host species, and showed similarity to related coronaviruses from China and European countries. A new coronavirus species was revealed among insectivores, which belonged to the *Alphacoronavirus* genus.

THE AIM OF THE STUDY

To study the diversity of coronaviruses among rodents and insectivores in the Altai Republic.

MATERIALS AND METHODS

Small mammals were captured on the northern coasts of Lake Teletskoye in the Altai Republic (51° 47´ N, 87° 18´ E). Animals were captured in accordance with the protocol and recommendations for safe work in compliance with methodological guidelines MU 3.1.1029-01, approved on April 6, 2001; cotton swabs were used to collect bat oral swabs; rectum pieces were placed in a solution with RNA Later (QIAGEN, Germany).

RNA isolation was performed using the RIBO-prep kit (Central Research Institute of Epidemiology, Russia) from lavages or rectal slices. 0.2 ml of sterile 0.9% NaCl solution was added to the tubes with lavages before isolation. Samples were screened by two-round reverse transcriptase-polymerase chain reaction (RT-PCR) using RevertAid Premium RNA polymerase (Thermo Fisher Scientific, USA), Hot Start Taq DNA polymerase (Sibenzyme, Russia) according to the protocol and with primers for the conserved region of the coronavirus RNA-dependent RNA polymerase (*RdRp*) gene (fragment 397 nucleotides) that have previously been described [12]. The nucleotide sequences of each amplicon chain were determined using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) on an ABI Prism 310 automatic analyser. A taxonomical identification of coronaviruses and their natural hosts was based on the determination and comparison with the GenBank database of the nucleotide sequences of viral genome fragments and the cytochrome b gene of the hosts' mitochondrial DNA.

Nucleotide sequence alignment was performed using the MUSCLE algorithm in MEGA X software (Mega Software, USA). The maximum likelihood (ML) method with the GRT + G + I evolutionary model was used to construct the phylogenetic tree. Calculations were performed for 500 repeats.

RESULTS AND DISCUSSION

In September 2022, 119 specimens of 10 species of small mammals were captured within the forest area located in the vicinity of the village of Artybash in the Altai Republic (Table 1). All captured animals were tested for the presence of coronavirus RNA.

3.8 % (2/52) of insectivores and 5.9 % (4/67) of rodents were positive for coronaviruses (Table 1). Viral RNA was revealed in 2 out of 11 Siberian shrew (*Crocidura sibirica*),

1 out of 5 bank voles (*Myodes glareolus*), 1 out of 15 red-backed voles (*Myodes rutilus*), 1 out of 20 field mice (*Apodemus agrarius*) and 1 out of 15 Korean field mice (*Apodemus peninsulae*).

Based on the results of phylogenetic analysis, the rodent-borne coronaviruses (*M. rutilus*, *M. glareolus*, *A. peninsulae* and *A. agrarius*) were classified in the subgenus *Embecovirus* of the genus *Betacoronavirus*. The shrew-borne virus detected in *C. sibirica*, was classified in the genus *Betacoronavirus* and is probably a representative of a new subgenus, as it differs significantly from other known coronaviruses (Fig. 1).

The nucleotide sequences of the RdRp gene fragment of new RNA isolates of coronaviruses identified in rodents, have a homology level of 86.7 % between isolates from animals of the genus *Myodes* and *Apodemus*, are identical for closely related species *M. rutilus/M. glareolus* and *A. peninsulae/A. agrarius* and demonstrate close similarity with previously published sequences of coronaviruses found in each of the host groups. Specifically, for the new coronavirus isolates from *M. glareolus/M. rutilus* (RtCoV/Mg-724/RUS/2022 and RtCoV/Mrut-816/RUS/2022), a similarity level of 95.8 % and 93.8–94.9 % was found with the RtCoV/Mrut-288/RUS/2021 RNA isolate identified from *M. rutilus* in the Novosibirsk region and in Germany (D_RMU10_1974/Myo_gla/GER and D_RMU10_1919/Myo_gla/GER), respectively. For Siberian isolates of coronavirus from the Altai Republic from *A. agrarius/A. peninsulae* (RtCoV/Ap-709/RUS/2022 and RtCoV/Aa-818/RUS/2022), a high level of homology was revealed (97.2 % and 98.3–98.6 %, respectively) both with RtCoV / Aa-528/RUS/2021 isolate from *A. agrarius* from the nearby Novosibirsk Region, and with geographically remote strains from China (RtAa/SX2014) and Germany (KS11_0997/Apo_agr/GER/2011), respectively.

The coronavirus identified in two specimens of the Siberian white-toothed shrew (ShrewCov/Cs-711/RUS/2022 and ShrewCov/Cs-764/RUS/2022) differs by more than 28.0 % in nucleotide sequence from other representatives of the genera *Alfacoronavirus* and *Betacoronavirus*. The highest level of difference (45.0 %) was found with coronavirus from another insectivore species, the common shrew (*Sorex araneus*) (ShrewCoV/Sa-314/RUS/2021), circulating in the nearby Novosibirsk region. In contrast to rodent-associated coronaviruses, coronaviruses from insectivores revealed higher genome variability. Specifically, the level of sequence differences obtained from two Siberian white-toothed shrew captured at the same site was 2.0 %. Considering the high level of variation in the conserved *RdRp* gene with representatives of other genera and species, it can be assumed that the RNA isolate from *C. sibiric*a is a representative of a new subgenus of the *Betacoronavirus* genus. This requires sequencing the full-length genome and analysing it according to the criteria of the international committee of viral taxonomy [22].

We have studied the diversity of coronaviruses circulating among rodents and insectivores in a natural focus located in the Altai Republic. Co-circulation of several coronavirus species has been shown for the same location, as was previously reported [21]. All the studied animals were captured in the vicinity of the Teletskiy scientific station of the Institute of Systematics and Ecology of Animals SB RAS (Siberian Branch of the Russian Academy of Sci-

TABLE 1

SPECIES COMPOSITION OF INSECTIVORES AND RODENTS TESTED FOR CORONAVIRUS RNAs

Carriers	Type	Coronavirus RNA+/studied
Insectivores	Common shrew (*Sorex araneus*)	0/39
	Taiga shrew (*Sorex isodon*)	0/1
	Laxmann's shrew (*Sorex caecutiens*)	0/1
	Siberian shrew *(Crocidura sibirica)*	2/11
Rodents	Korean field mouse (*Apodemus peninsulae*)	1/15
	Striped field mouse (*Apodemus agrarius*)	1/20
	Root vole (*Alexandromys oeconomus*)	0/3
	Grey-red-backed vole (*Myodes rufocanus*)	0/9
	Red-backed vole (*Myodes rutilus*)	1/15
	Bank vole (*Myodes glareolus*)	1/5

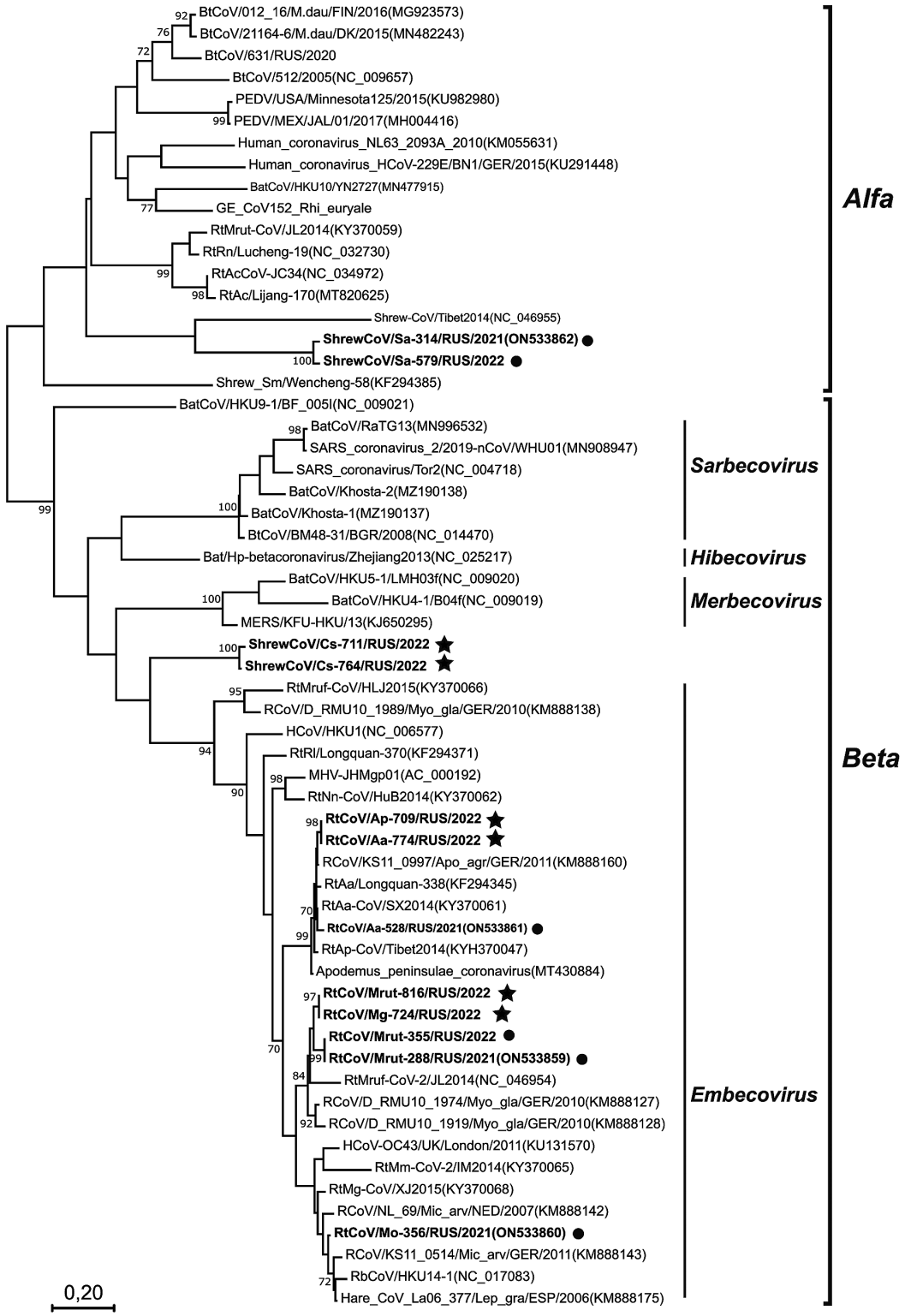

FIG. 1.

Phylogenetic analysis of the nucleotide sequences of the RdRp gene fragment of coronaviruses corresponding to positions 15429-15825 of the HKU-1 isolate (Genbank NC_006577). New coronaviruses obtained from Myodes glareolus (RtCoV/Mg-724/RUS/2022), Myodes rutilus (Rt CoV/Mrut-816/RUS/2022), Apodemus peninsulae (RtCoV/Ap-709/RUS/2022), Apodemus agrarius (RtCoV//Aa-818/RUS/2022) and Crocidura sibirica (ShrewCoV/Cs-711/RUS/2022, ShrewCoV/Cs-764/RUS/2022), were compared with known CoVs belonging to the genera Alphacoronavirus and Betacoronavirus. The tree is constructed using the maximum likelihood method and the GRT + G + I evolution model. Support indices were calculated for 500 repetitions, and support indices (> 70 %) are represented at the respective nodes. The scale bar indicates the number of nucleotide substitutions per site. New isolates are highlighted in bold and asterisk, isolates from the Novosibirsk region are highlighted in bold and circle

ences) within a forest area of no more than 0.2 km^2. Co-circulation of two rodent-borne coronaviruses and one coronavirus carried by an insectivorous host, the Siberian shrew, has been demonstrated in a limited area.

Coronaviruses associated with natural carriers belonging to genus *Myodes* and *Apodemus* have previously been reported to be classified into two genera – Alfacoronavirus and Betacoronavirus [15]. New Siberian isolates from rodents classified in the *Embecovirus* subgenus of the genus *Betacoronavirus* were characterised by high (93.8–95.8 % and 97.2–98.3 %) homology with previously known isolates in each group, found over a wide area including Europe (Germany), Siberia (Novosibirsk region) and China, with sequences from a single capture site in the Altai Republic being identical. These findings indicate relatively high stability of coronavirus genomes compared to other RNA-containing viruses associated with rodents. Accordingly, the difference in *RdRp* gene sequences in hantaviruses (family Hantaviridae, genus *Orthohantavirus*) found in the same host species from geographically distant regions of Siberia and Europe was more than 20 % [23]. A higher level of variation in partial nucleotide sequences of the *RdRp* gene was found among coronaviruses from insectivores. The level of sequence difference from two specimens of Siberian shrews was 2.0 %, which is comparable to the level of difference between Seewis hantavirus isolates from the same location [24].

CONCLUSION

Three different coronaviruses were detected among 4 species of rodents and one species of insectivores living together in the natural biotope in the Altai Republic. Coronaviruses from rodents (*M. glareolus/M. rutilus* and *A. agrarius/A. peninsulae*) are classified in the genus *Betacoronavirus* subgenus *Embecovirus*, coronavirus from an insectivore (*C. sibirica*) is classified in the genus *Betacoronavirus* and is assumed to belong to a new subgenus. The genomes of rodent-associated coronaviruses were revealed to be highly stable.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Liudmila N. Yashina – Dr. Sc. (Biol.), Leading Research Officer at the Department of Genomic Research, State Research Center of Virology and Biotechnology "Vector", e-mail: yashina@vector.nsc.ru, https://orcid.org/0000-0003-2844-7835

Sergey A. Abramov – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Ecology of Vertebrate Communities, Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences, e-mail: gterio@gmail.com, https://orcid.org/0000-0001-7921-6696

Natalia A. Smetannikova – Cand. Sc. (Med.), Senior Research Officer at the Department of Genomic Research, State Research Center of Virology and Biotechnology "Vector", e-mail: smetannikova@vector.nsc.ru, https://orcid.org/0000-0002-5082-8071

Boris S. Malyshev – Research Officer at the Department of Genomic Research, State Research Center of Virology and Biotechnology "Vector", e-mail: malyshevb@vector.nsc.ru, https://orcid.org/0000-0003-3004-3020

Anton V. Krivopalov – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Parasitology, Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences, e-mail: krivopalov@gmail.com, https://orcid.org/0000-0003-4824-6061

Tamara A. Dupal – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Ecology of Vertebrate Communities, Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences, e-mail: gf@eco.nsc.ru, https://orcid.org/0000-0002-4487-1815

ONLINE SERVICE FOR INTERPRETATION OF THE RESISTANCE PREDICTION RESULTS TO BEDAQUILINE BY THE MOLECULAR DATA

Sinkov V.V. [1],
Kondratov I.G. [1],
Ogarkov O.B. [1],
Zhdanova S.N. [1],
Noskov A.P. [2],
Khromova P.A. [1],
Orlova E.A. [1],
Labygina A.V. [1],
Rychkova L.V. [1],
Kolesnikova L.I. [1]

[1] Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)
[2] Irkutsk Regional Clinical Tuberculosis Hospital (Tereshkovoy str. 59, Irkutsk 664039, Russian Federation)

Corresponding author:
Oleg B. Ogarkov,
e-mail: obogarkov@mail.ru

ABSTRACT

Background. *Bedaquiline is a new and promising anti-tuberculosis drug, however resistance can develop with long-term use. This is mainly due to mutations in the atpE and mmpR genes in M. tuberculosis (MTB).*
The aim of the work. *The aim of the research was to test a system for automated interpretation of results for predicting resistance to bedaquiline by the molecular data.*
Materials and methods. *DNA was isolated from strains of M. tuberculosis in the Irkutsk region and Yakutia. The total quantity of DNA samples was 27 strains from Yakutia and 21 strains from the Irkutsk region. The study of MBT genomes was carried out on the DNA previously obtained by the authors in the territories of the Irkutsk region (n = 5), Yakutia (n = 4), Buryatia (n = 3), Zabaykalskiy kray (n = 4) and the Far East (n = 8). We used the BSATool program to detect bedaquiline resistance based on Sanger sequencing and genomic data. Sanger sequencing analyzed the atpE and mmpR genes, and whole genome sequencing examined mutations in the same sequences, as well as additionally in mmpL5, mmpS5, Rv0678, Rv1979c, and pepQ.*
Results. *Complete agreement between the phenotypic and genotypic analysis of resistance to bedaquiline was found for three strains from Yakutia. One genome with significant mutations associated with resistance to bedaquiline was identified. A conclusion was made about the relatively low prevalence of mutations that may induce resistance to this antibiotic, which coincides with the data of other studies in Russia. A conclusion was made about the importance of molecular analysis of target genes with subsequent detection of resistance to bedaquiline in silico.*

Key words: *bedaquiline, sequencing, resistance, genes, atpE, mmpR, mmpL5, mmpS5, Rv0678, Rv1979c, pepQ*

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ОНЛАЙН-СЕРВИС ДЛЯ ИНТЕРПРЕТАЦИИ РЕЗУЛЬТАТОВ ПРИ ПРОГНОЗИРОВАНИИ УСТОЙЧИВОСТИ К БЕДАКВИЛИНУ ПО МОЛЕКУЛЯРНО-БИОЛОГИЧЕСКИМ ДАННЫМ

Синьков В.В. [1],
Кондратов И.Г. [1],
Огарков О.Б. [1],
Жданова С.Н. [1],
Носков А.П. [2],
Хромова П.А. [1],
Орлова Е.А. [1],
Лабыгина А.В. [1],
Рычкова Л.В. [1],
Колесникова Л.И. [1]

[1] ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)
[2] ОГБУЗ «Иркутская областная клиническая туберкулёзная больница» (664039, г. Иркутск, ул. Терешковой, 39, Россия)

Автор, ответственный за переписку:
Огарков Олег Борисович,
e-mail: obogarkov@mail.ru

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РЕЗЮМЕ

Обоснование. *Бедаквилин – новый и многообещающий противотуберкулёзный препарат, однако при длительном лечении к нему развивается устойчивость. Это связано преимущественно с мутациями в генах atpE и mmpR у M. tuberculosis (МБТ).*

Цель работы. *Апробация системы автоматизированной интерпретации результатов при прогнозировании устойчивости к бедаквилину на основе молекулярно-биологических данных.*

Материалы и методы. *ДНК выделяли из штаммов M. tuberculosis, циркулировавших в Иркутской области и Республике Саха (Якутии). Общее количество исследованных ДНК составило 27 штаммов из Якутии и 21 штамм из Иркутской области. Исследование геномов МБТ было проведено на ДНК штаммов, полученных авторами ранее на территориях Иркутской области (n = 5), Республики Саха (Якутия) (n = 4), Республики Бурятия (n = 3), Забайкальского края (n = 4) и Дальнего Востока (n = 8). Для выявления устойчивости к бедаквилину на основе нуклеотидной последовательностей генов и геномных данных мы использовали программу BSATool. При использовании секвенирования по Сэнгеру анализировались гены atpE и mmpR, при полногеномном секвенировании исследовались мутации в этих же последовательностях, а также дополнительно в mmpL5, mmpS5, Rv0678, Rv1979c и pepQ.*

Результаты. *Обнаружено полное соответствие фенотипических и генотипических результатов оценки устойчивости к бедаквилину для трёх штаммов из Якутии. Кроме того, при анализе геномных данных обнаружен один геном со значимыми мутациями, способными вызвать устойчивость к бедаквилину. Делается вывод об относительно низком распространении мутаций, способных вызвать устойчивость к этому антибиотику, что совпадает с данными других исследователей в России. Сделано заключение о важности молекулярно-биологического анализа генов-мишеней с последующим выявлением устойчивости к бедаквилину in silico.*

Ключевые слова: *бедаквилин, секвенирование, резистентность, гены, atpE, mmpR, mmpL5, mmpS5, Rv0678, Rv1979c, pepQ*

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Tuberculosis is an infectious disease caused by pathogenic mycobacteria belonging to the class Actinobacteria, order Actinomycetales, family Mycobacteriaceae, forming the *Mycobacterium tuberculosis* complex group [1]. In 2019, the World Health Organization (WHO) reported 10 million new cases and 1.2 million deaths [2]. However, the emergence and spread of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* up to half a million annually [3] requires the introduction of new anti-TB drugs into treatment regimens. Bedaquiline is a new and promising antituberculosis drug, but as with other drugs, it is possible that *M. tuberculosis* (MTB) may develop resistance to it with long-term treatment. Specifically, mutations in the *atpE* gene prevent drug interaction with its target, ATP synthase, and mutations in the efflux pump repressor *mmpR* (*Rv0678*) lead to accelerated drug evacuation from the microbial cell [4–6]. Despite the involvement of a significant number of genes in resistance to bedaquiline and clofazimine (including cross-resistance), mutations in the *atpE* and *mmpR* genes (*Rv0678*) are determinant in the majority of clinical isolates [7]. Considering these results, a Sanger sequencing of these genes was used to analyse the developed software package.

An online service for automated interpretation of sequencing data and prediction of pyrazinamide resistance has been previously developed by us [8]. The service is available at https://bsatool.ru. In the current study, we present an extension of the capabilities of the BSATool software package for automated interpretation of results in predicting bedaquiline resistance based on molecular biological data, including Sanger sequencing and whole genome sequencing.

MATERIALS AND METHODS

The strains were obtained from the bacteriological laboratories of the Irkutsk Regional Clinical Tuberculosis Hospital (IRCTB) and E.N. Andreev Scientific and Practical Centre "Phthisiatry" (SPC "Phthisiatry"). Phenotypic sensitivity of MTB isolates to antituberculosis drugs (ATDs) was determined by absolute concentrations on Levenshtein – Jensen medium (IRCTB and SPC "Phthisiatry") and on Middlebrook 7H9 medium using an automated system Bactec MGIT 960 (Becton, Dickinson and Company, USA), including sensitivity to bedaquiline (SPC "Phthisiatry"). Strains were inactivated *in situ*. DNA isolation was performed according to the previously described method [9].

The total number of DNA samples examined included 27 strains from Yakutia and 21 strains from the Irkutsk region. The study of MBT genomes was carried out on the MBT strains previously obtained by the authors in the territories of the Irkutsk region ($n = 5$), Yakutia ($n = 4$), Buryatia ($n = 3$), Zabaykalskiy kray ($n = 4$) and the Far East ($n = 8$). Therefore, our study included a set of DNA samples representing different regions and territories. The structure of polymerase chain reaction (PCR) primers for the amplification of *atpE* and *mmpR* genes was developed independently by the authors. The following primers were used for the *atpE* gene: 1305F 5'-*TCGAAGAGGAACACCACTAG* and 1305R 5'-*GGACAATCGCGCTCACTTC*. The primers Bdq678F 5'-*CACGCTTGAGAGTTCCAATCA* and Bdq678R 5'-*ACCGCATCAACAAGGAGTGA* were used for the *mmpR* (Rv0678) gene. These oligonucleotides were designed to amplify PCR fragments of 368 and 679 base pairs, respectively.

PCR parameters were set, according to previously published protocols [8]. Sanger sequencing was performed using a domestic genetic analyzer Nanofor-05 (Syntol, Russia). Genomic libraries were prepared using DNA Flex kit (Illumina, USA). Full genome sequencing of samples was performed on NextSeq 550 sequencer (Illumina, USA) also using reagents v. 2.5 and flow cell (high output) at 300 cycles.

Primary data processing included removal of short low-quality sequences and technical fragment cut-offs and was performed according to previously published methods [10]. Sanger sequencing and full-genome sequencing were performed at the Center for Development of Progressive Personalized Health Technologies (shared research facility of the Scientific Centre for Family Health and Human Reproduction Problems (Irkutsk).

To reveal resistance to bedaquiline by Sanger sequencing, we used the BSATool software [8], which analysed mutations in *atpE* and *mmpR*. To reveal resistance to bedaquiline, mutations in the same genes (*atpE* and *mmpR*) and additionally in *mmpL5*, *mmpS5*, *Rv0678*, *Rv1979c* and *pepQ* were analysed using BSATool genomic data. Based on the above analyses, potential resistance to bedaquiline was identified in an *in silico* model. The clinical significance of the detected mutations in causing drug resistance was assessed according to the mutation catalogue recommended by the World Health Organization as a reference database [11].

RESULTS

Analysis of Sanger sequencing results in manual mode and by the BSATool software system

Of the 27 strains obtained from the Republic of Sakha (Yakutia), 9 were classified as extensively drug-resistant strains or their precursors (pre-XDR), 10 were sensitive to all ATDs, and the rest showed multidrug resistance [11]. Resistance to bedaquiline was found in only three pre-XDR strains.

When analysed by Sanger sequencing and using the BSATool service, significant mutations with the potential to cause resistance to bedaquiline were revealed in the same three strains. Based on the results of microbiological studies, these strains were registered as XDR.

Among the 21 strains from Irkutsk, 10 were classified as pre-XDR and the remaining 11 strains showed multidrug resistance. Among all these strains, only two had significant mutations that can induce resistance to bedaquiline, both in manual Sanger sequencing analysis

and using the BSATool service. Notwithstanding, due to the lack of a methodology for determining resistance to bedaquiline in the bacteriological laboratory of the IRCTB, these strains were not assigned XDR status.

Figure 1 shows an example of DNA analysis of four strains using the BSATool software package, one of which contains a mutation that can induce resistance to bedaquiline.

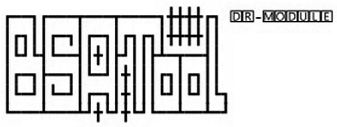
Analysis of the results of whole genome sequencing by BSATool software package

Manual analysis of the whole-genome data was not feasible due to the large amount of information. The study analysed the genome of 24 MTB strains, including those from the Irkutsk Region, the Republic of Sakha (Yakutia), the Republic of Buryatia, the Trans-Baikal Territory and the Far East. A significant mutation 2223444C/T in the *Rv1979* gene was only revealed in one strain, which belongs to the most virulent subtype B0/W148 of the Beijing genotype [12]. It is important to emphasize that the remaining strains analyzed by whole-genome sequencing did not show resistance to bedaquiline as part of *in silico* prediction.

DISCUSSION

Assessment of resistance to anti-TB drugs using molecular biological methods requires an individual approach depending on the prevalence of clinically relevant mutations. The hotspots for rifampicin are centered on a small 81 bp (base pair) long stretch of DNA and the most feasible method is to reveal them by real-time PCR. This method has already been successfully used in the practice of medical diagnostics for almost a decade.

For other ATDs, the situation with clinically relevant mutations is not as favourable, which requires the use of sequencing of multiple gene nucleotide sequences. The use of software systems that can reveal significant mutations in the results of whole-genome sequencing or Sanger sequencing significantly expands the possibilities of using these molecular biological methods in clinical practice. The relatively low frequency of revealed mutations causing resistance to bedaquiline is consistent with the results of studies conducted by colleagues in the European part of Russia [13]. All the mutations identified were observed in patients receiving long-term bedaquiline treatment as part of MDR/XDR tuberculosis chemotherapy regimens.



BSATool - Bacterial SNP Annotation Tool
(c) V.Sinkov, Irkutsk, Russia, 2017-2023
Version: 0.3.170723

Query	Query name	Query start	Query end	Query lenght	Query direction	Mutations	Intersected regions
Q1	825F.fa	1461033	1461317	284	-->	0	IGR_1460997_1461044,Rv1305,IGR_1461291_1461320
Q2	825R.fa	1461007	1461277	270	<--	0	IGR_1460997_1461044,Rv1305
Q3	850F.fa	1461039	1461311	272	-->	0	IGR_1460997_1461044,Rv1305,IGR_1461291_1461320
Q4	850R.fa	1461011	1461303	292	<--	0	IGR_1460997_1461044,Rv1305,IGR_1461291_1461320
Q5	860F.fa	1461032	1461317	285	-->	1	IGR_1460997_1461044,Rv1305,IGR_1461291_1461320
Q6	860R.fa	1461017	1461266	249	<--	1	IGR_1460997_1461044,Rv1305
Q7	870F.fa	1461043	1461320	277	-->	0	IGR_1460997_1461044,Rv1305,IGR_1461291_1461320
Q8	870R.fa	1461006	1461260	254	<--	0	IGR_1460997_1461044,Rv1305

Query	Query name	Locus	Gene	Genome position(s)	Type	Mutation	Codon	Amino Acid	Change	Description	Drug	DR	DR Info
Q1	825F.fa	-	-	-	-	-	-	-	-	-	-	-	-
Q2	825R.fa	-	-	-	-	-	-	-	-	-	-	-	-
Q3	850F.fa	-	-	-	-	-	-	-	-	-	-	-	-
Q4	850R.fa	-	-	-	-	-	-	-	-	-	-	-	-
Q5	860F.fa	Rv1305	atpE	1461242	SNP	c.198C>G	Caa/Gaa	Q66E	missense	Probable ATP synthase C chain AtpE	BDQ	R	I66M
Q6	860R.fa	Rv1305	atpE	1461242	SNP	c.198C>G	Caa/Gaa	Q66E	missense	Probable ATP synthase C chain AtpE	BDQ	R	I66M
Q7	870F.fa	-	-	-	-	-	-	-	-	-	-	-	-
Q8	870R.fa	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 1.
Analysis of the results of Sanger sequencing of the atpE gene for two strands in four different strains: a significant mutation associated with resistance to bedaquiline was revealed in strain 860; no resistance-associated mutations were revealed in the other strains

CONCLUSION

Molecular biological analysis of nucleotide sequences of target genes followed by *in silico* resistance assessment against bedaquiline using the BSATool automated analysis system (publicly available) may be recommended primarily for laboratories performing Sanger sequencing. The low frequency of mutations associated with resistance to bedaquiline may be explained by the recent use of the drug in the treatment of patients and, consequently, by the small number of MTB strains resistant to bedaquiline. The validation of the proposed approaches for predicting bedaquiline resistance in clinical samples from patients with repeated courses of MDR/XDR tuberculosis treatment confirms the reliability of the obtained data.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Vyacheslav V. Sinkov – Cand. Sc. (Med.), Senior Research Officer at the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: vsinkov@gmail.com, https://orcid.org/0000-0003-3396-9590

Ilya G. Kondratov – Research Officer at the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: kondratovig@mail.ru, https://orcid.org/0000-0002-2631-4724

Oleg B. Ogarkov – Dr. Sc. (Med.), Head of the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: obogarkov@mail.ru, https://orcid.org/0000-0002-3168-1983

Svetlana N. Zhdanova – Dr. Sc. (Med.), Leading Research Officer at the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: svetnii@mail.ru, https://orcid.org/0000-0001-7160-9700

Alexander P. Noskov – Bacteriologist, Irkutsk Regional Clinical Tuberculosis Hospital, e-mail: alex.noskoff@mail.ru

Polina A. Khromova – Junior Research Officer at the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: polina.and38@gmail.com, https://orcid.org/0000-0002-6449-5060

Elizaveta A. Orlova – Postgraduate, Junior Research Officer at the Institute of Epidemiology and Microbiology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: elizaveta.a.orlova@gmail.com, https://orcid.org/0000-0003-2169-0242

Albina V. Labygina – Dr. Sc. (Med.), Research Officer at the Laboratory of Gynaecological Endocrinology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: albinalab2212@mail.ru, https://orcid.org/0000-0001-8190-6143

Lyubov V. Rychkova – Dr. Sc. (Med.), Professor of the RAS, Corresponding Member of the RAS, Director, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-0117-2563

Lyubov I. Kolesnikova – Dr. Sc. (Med.), Professor of the RAS, Member of the RAS, Academic Advisor, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0003-3354-2992

CHARACTERISTICS OF TICK-BORNE INFECTIONS IN THE UNDEREXPLORED AREAS OF THE TRANS-BAIKAL TERRITORY

Lagunova E.K.,
Khasnatinov M.A.,
Danchinova G.A.

Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)

Corresponding author:
Maxim A. Khasnatinov,
e-mail: khasnatinov@gmail.com

ABSTRACT

Background. *Infections that are transmitted to humans through the bites of ixodid ticks remain a significant problem for public healthcare. However, in many regions endemic for tick-borne diseases, the diversity and prevalence of infections transmitted by ticks are still underexplored.*

The aim of the study. *To characterize the modern diversity and prevalence of tick-borne pathogens in the ecosystems of the valley of the Chikoy River (Trans-Baikal Territory, Russian Federation).*

Materials and methods. *Real-time polymerase chain reaction was used to examine 48 imagoes of Ixodes persulcatus ticks, one Haemaphysalis concinna female tick and 38 small mammals specimens for infection with seven tick-borne pathogens.*

Results. *The H. concinna tick tested negative for all studied pathogens. In I. persulcatus ticks, the prevalence of infection with Borrelia burgdorferi s.l. comprised 39.5 %, Anaplasma phagocytophilum – 16.7 %, B. miyamotoi – 8.3 % and Ehrlichia sp. – 2.1 %. Tick-borne encephalitis virus (TBEV), Rickettsia sibirica and R. heilongjiangensis were not detected in I. persulcatus. Four species of vertebrate hosts of ticks and tick-borne infections were found: Myodes rufocanus (44.7 %) Apodemus peninsulae (39 %), Microtus oeconomus (13.2 %) and M. rutilus (2.6 %). In rodent populations, the prevalence of TBEV infection comprised 5.3 %, B. burgdorferi s. l. – 39.5 %, B. miyamotoi – 28.9 %, Ehrlichia sp. – 21.1 %, A. phagocytophilum – 18.4 %.*

Conclusion. *The widespread distribution of taiga ticks, the presence of numerous populations of competent vertebrate hosts of infections, and the high prevalence of infections among vertebrate and invertebrate hosts indicate that active natural foci of tick-borne encephalitis, Lyme disease, tick-borne relapsing fever caused by B. miyamotoi, human granulocytic anaplasmosis, and human monocytic ehrlichiosis (HME) are present in the ecosystems of Chikoy River valley.*

Key words: *the Trans-Baikal Territory, Borrelia, tick-borne encephalitis virus, Anaplasma phagocytophilum, Riskettsia, Ixodes persulcatus*

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ХАРАКТЕРИСТИКА КЛЕЩЕВЫХ ИНФЕКЦИЙ В МАЛОИЗУЧЕННЫХ РАЙОНАХ ЗАБАЙКАЛЬСКОГО КРАЯ

Лагунова Е.К.,
Хаснатинов М.А.,
Данчинова Г.А.

ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)

Автор, ответственный за переписку:
Хаснатинов Максим Анатольевич,
e-mail: khasnatinov@gmail.com

РЕЗЮМЕ

Обоснование. *Инфекции, передающиеся человеку при укусах иксодовых клещей, остаются актуальной проблемой здравоохранения. Однако во многих частях нозоареала разнообразие и распространённость клещевых инфекций остаются недостаточно исследованными.*

Цель исследования. *Охарактеризовать современное разнообразие и распространённость возбудителей клещевых инфекций в долине р. Чикой (Забайкальский край, Россия), входящей в буферную зону Байкальской природной территории.*

Материалы и методы. *С помощью полимеразной цепной реакции в реальном времени на заражённость семью возбудителями клещевых инфекций исследованы 48 имаго клещей Ixodes persulcatus, 1 особь Haemaphysalis concinna и 38 особей мелких млекопитающих.*

Результаты. *Клещ H. concinna не был заражён ни одним из исследуемых патогенов. Зараженность I. persulcatus Borrelia burgdorferi s. l. составила 39,5 %, Anaplasma phagocytophilum – 16,7 %, B. miyamotoi – 8,3 % и Ehrlichia sp. – 2,1 %. Вируса клещевого энцефалита (ВКЭ), Rickettsia sibirica и R. heilongjiangensis в таёжных клещах не обнаружено. Выявлены 4 вида позвоночных хозяев клещей и инфекций: Myodes rufocanus (44,7 %) Apodemus peninsulae (39 %), Microtus oeconomus (13,2 %) и M. rutilus (2,6 %). В популяциях мелких млекопитающих зараженность ВКЭ составила 5,3 %, B. burgdorferi s. l. – 39,5 %, B. miyamotoi – 28,9 %, Ehrlichia sp. – 21,1 %, A. phagocytophilum – 18,4 %.*

Заключение. *Повсеместное распространение таёжных клещей, наличие многочисленных популяций компетентных позвоночных хозяев инфекций, а также высокие показатели заражённости позвоночных и беспозвоночных хозяев свидетельствуют о широком распространении в долине р. Чикой активных природных очагов клещевого энцефалита, болезни Лайма, клещевой возвратной лихорадки, вызываемой B. miyamotoi, гранулоцитарного анаплазмоза и моноцитарного эрлихиоза человека.*

Ключевые слова: *Забайкальский край, Borrelia, вирус клещевого энцефалита, Anaplasma phagocytophilum, Riskettsia, Ixodes persulcatus*

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INTRODUCTION

Zoonotic infections are still recognized as one of most significant threats to the human health in Russian Federation. This is particularly true for infections transmitted by ixodid ticks due to the wideness of spread and the severity of the illnesses. In the Asian part of the Russian Federation, the main vector of many tick-borne infections are *Ixodes persulcatus* ticks (Schulze, 1930) [1].

The most epidemically significant causative agents of tick-borne diseases (TBD) are tick-borne encephalitis virus (TBEV), Lyme disease agent *Borrelia burgdorferi* sensu lato, the agent of the tick-borne relapsing fever *B. miyamotoi* a few species of rickettsiae, anaplasmas, ehrlichiae and other pathogens [2]. Ticks can be infected either by a single human pathogen (mono-infection) or simultaneously by two or more species of pathogens (co-infection). Mono- and co-infections are clinically distinctive depending on the combination of pathogens. This requires the development of new approaches to the diagnosis, prevention and treatment of known tick-borne infections, including co-infections [3, 4].

The main source of information for assessing the epidemiological situation is active monitoring of natural foci of tick-borne infections, based on direct evaluation of the number of tick vectors and mammals – reservoir hosts of infections and tick feeders in the ecosystem, as well as evaluation of the prevalence of infection with the studied pathogens. Active monitoring requires highly qualified personnel and is labor-intensive and time-consuming. As a result, in many territories that are endemic for TBD, there is a lack of information about the prevalence and diversity of tick-borne infections, as well as the structure and risk assessment of their natural foci. Despite the obvious advantages of this approach, it has a number of significant drawbacks. This method, for example, does not consider a number of important aspects of the infection process, such as multiplexing of several pathogens in one parasitic system, the intensity of contact between people and tick vectors, the socio-demographic state of the population, and migration processes in human and wildlife populations [5]. Besides, in order to obtain reliable data during active monitoring, it is necessary to study large numbers of vectors and feeders (ticks and vertebrates) at the same time at sampling key sites located in different landscapes The study should be performed using unified methods of detection and identification of pathogens for the entire sample of materials collected during field expeditions [6, 7]. As a result, in many areas endemic for tick-borne infections, the peculiarities of the spread and circulation of pathogens in nature remain poorly studied.

One of such areas is the Krasnochikoysky district of the Trans-Baikal Territory, which has the highest incidence rates of tick-borne encephalitis and Lyme disease [8]. Despite this, research on tick-borne infections in these areas has previously been sporadic and was mainly focused on tick-borne encephalitis virus and Lyme disease agents, while no information is available concerning the circulation of other pathogens transmitted by ixodid ticks. The Chikoy is one of the largest rivers in the Lake Baikal catchment area, and the Chikoy valley along with the adjacent ranges of the Khentii Mountains is part of the Baikal Natural Area. Large areas of indigenous cedar forest unaffected by human activity, peculiar flora and fauna, unique natural objects, and deposits of natural resources (uranium ores) predetermine the potential for further recreational, economic and industrial development of the Chikoy valley [9]. Given the foregoing, characterization of the current situation in natural foci of TBD seems to be a significant task both scientifically and practically.

THE AIM

To characterize the current diversity and prevalence of causative agents of tick-borne infections in the Chikoy River valley (Krasnochikoysky District, Trans-Baikal Territory, Russian Federation).

MATERIALS AND METHODS

Materials

Ixodid ticks were collected from vegetation in the most common biotopes of the study area using flannel flag. The abundance of the ticks was evaluated using the standard methods [10] and expressed as the number of ticks per flag per kilometer of the route. The captured ticks were delivered to the laboratory alive. Information about each tick was registered in the information-analytical system "Field ticks" [11]. Small mammals were captured using Sherman live traps on survey lines. The abundance of animals was expressed as a number of specimens per trap per day. Animals were euthanized at the place of capture in compliance with the "Ethical Principles for Medical Research Involving Human Subjects" [12]. For each captured animal, species, sex, age group (juveniles or adults), and infestation with ectoparasites were individually determined. Afterwards the tissue sampling of brain, spleen, kidney and lung was performed. Tissue samples were stored in liquid nitrogen until delivery to the laboratory and then at –80 °C until study.

Preparation of tick and mammalian organ suspensions

Each tick was individually washed in 70 % ethanol and dried on filter paper, and then the species, sex and condition of the tick were determined. The tick species was identified morphologically using the key guide of the USSR ixodid tick fauna [13]. Prepared ticks were homogenized using a TissueLyzer II automatic homogenizer (QIAgen, Germany) and sterile 3 mm diameter tungsten carbide beads. Homogenates were resuspended in 300 µl of sterile phosphate buffered saline PBS; (pH = 7.4) and examined immediately after preparation.

The species of small mammals was identified morphologically in accordance with the key guide of the USSR rodent fauna [14]. The infestation of animals by ixodid ticks was assessed based on the index of abundance (IA; average number of ticks per specimen) and the index of occurrence (IO; proportion of animals in the sample on which ixodid ticks were found).

Animal tissue samples (30–50 mg) were homogenized using a TissueLyzer II and chilled sterile 5 mm steel beads. Homogenates were resuspended in 300 µl of chilled sterile PBS (pH = 7.4) and examined immediately after preparation.

Nucleic acids extraction

Total RNA/DNA was extracted from individual ticks or tissue samples. Briefly, 100µl of suspension was purified using RealBest Extraction 100 or RealBest UniMag kits (VectorBest, Novosibirsk) and KingFisher Flex (Thermo Fisher Scientific, Finland) magnetic particle processor according to the manufacturer's instructions. The resulting nucleic acids were dissolved in 300 µl of elution buffer.

Real-time PCR

Polymerase chain reaction (PCR) with real-time hybridisation-fluorescence detection was used to reveal causative agents of tick-borne infections. Reagent kits "RealBest DNA *Borrelia burgdorferi s. l.*/RNA VKE", "RealBest DNA *Anaplasma phagocytophilum/Ehrlichiamuris, Ehrlichia chaffeensis*", "RealBest DNA *Borrelia miyamotoi*" and "RealBest DNA *Rickettsia sibirica/Rickettsia heilongjiangensis*" (VectorBest, Novosibirsk). Since *E. muris* and *E. chaffeensis* were detected in a single PCR reaction without species identification, the identified Ehrlichiae were hereafter designated as *Ehrlichia*sp.

Fifty microliters of DNA/RNA preparation was used as a matrix for reverse transcription PCR. Reaction and real-time results were recorded using a CFX C1000 Touch amplifier (BioRad, USA) according to the kit manufacturer's instructions. BioRad CFX Manager 3.1 software (BioRad, USA) was used to process and analyze the results.

Quantitative PCR

Quantification of *Borrelia* sp. was performed using multiplex quantitative PCR (qPCR) targeting the *16S* rRNA gene [15]. Briefly, the *16S rRNA* gene fragments of *B. burgdorferi* sensu lato (strain B31) and *B. miyamotoi* (strain HT31) were PCR amplified using BspF-16s [5`-GCTGTAAACGATGCACACTTGGT-3`] and BspR-16s [5`- GGCGGCACACTTAACACGTTAG-3`] primers. The obtained PCR fragments (70 nucleotide bases in length) were independently cloned in the plasmid vector pCR4-TOPO (Thermo Fisher Scientific, USA) and grown in competent DH5α strain of *Escherichia coli* (Nippon Gene, Japan) as described previously [15]. The number of spirochetes in the samples was estimated using calibration curve produced by serial tenfold dilution of standard DNA samples of the corresponding Borrelia spp. and expressed as log10 genome copies per tick. PCR was performed in a reaction volume of 25 µL. The reaction mixture contained 1U Taq polymerase HSTaq (Eurogen), 2.5 µl of DNA template, primers BspF-16s and BspR-16s at a concentration of 900 nM each and probes FAM-LD [5`-FAM-TTCGGTACTAACTTTTAGTTAA-BHQ1-3`] and VIC-RF [5`-R6G-CGGTACTAACCTTTCGATTA-BHQ1-3`] at a concentration of 200 nM each. PCR conditions were set up as follows: an initial cycle at 50 °C for 2 min, followed by a cycle at 95 °C for 2 min, then 45 cycles of 95 °C for 15 s and 63 °C for 60 °C. The fluorescence was measured at the 63 °C stage of every cycle and compared to the calibration curve readings, with the FAM channel determining the presence and concentration of B. burgdorferi s. l. DNA, and the VIC channel – the presence and concentration of B. miyamotoi DNA..

Statistical methods of data processing

Sex ratio and infection prevalence were assessed as the proportion of infected specimens (in percent) and 95% confidence intervals (95% CI) were calculated.

To assess associations between TBEV infections with *B. burgdorferi* s. l. l., *B. miyamotoi*, *A. phagocytophilum* and *Ehrlichia* sp. in ticks, 2×2 contingency tables were constructed for each pair of microorganisms [16]. In this case, infected and uninfected tick conditions were taken as binary determinants. Analysis of variance based on Fisher's *F*-criterion [17] was used to assess the statistical significance of differences between group mean values.

Statistical analyses were performed using MS Excel software (Microsoft Corp., USA), MaxStat Light and R version 4.0.2 (R Foundation, Austria). Statistical significance was set at 0.95. All tests of statistical significance were two-tailed. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The tick and animal surveys and sample collection were conducted between the 3rd and 10th of July, 2021 during a field survey along the Chikoy River valley in the Republic of Buryatia and Krasnochikoysky District of the Trans-Baikal Territory. A total of 13 sampling sites were studied (Fig. 1). The survey routes for tick abundance assessment and sample collection comprised 15.7 km. The survey of small mammal's abundance and specimen collection resulted in 455 traps-nights. In total, 49 specimens of ixodid ticks and 38 specimens of rodents were collected. Besides this, 116 larvae and nymphs of taiga ticks were found on small mammals, but were excluded from PCR tests.

Ixodid tick abundance ranged from low to moderate and ranged from 0.4 to 13 individuals per flag/km. Considering the suboptimal survey season (early July), such tick abundance suggest that there are natural foci of tick-borne infections with medium and high tick activity in the surveyed area. The highest tick numbers were associated with mixed grass pine-birch forests with spiraea and rosehip as underbrush. One tick captured at survey point No. 7 (Fig. 1) was identified as a female *Haemaphysalis concina*

(Koch, 1844). The remaining 48 ticks were identified as *Ixodes persulcatus* (Shulze, 1930). All ticks were imagoes, with a sex ratio of 0.6 males per female.

Relative abundance of small mammals ranged from 10 to 21.5 exv./100 trap-nights, with no animals captured at three locations. Species diversity was represented by four predominant species: Korean field mouse *Apodemus peninsulae* (Thomas, 1907), grey red-backed vole *Myodes rufocanus* (Sundevall, 1846), tundra vole *Microtus oeconomus* (Pallas, 1776), and grey red-backed vole *Myodes rutilus* (Pallas, 1779). *M. rufocanus* (44.7 %) and *A. peninsulae* (39.5 %) were the most common species captured, whereas *M. oeconomus* and *M. rutilus* were scarce (13.2 % and 2.6 % of the sample, respectively).

I. persulcatus larvae were found only on rodents of two species: *A. peninsulae* and *M. rufocanus*, with *A. peninsulae* playing a slightly more significant role in the feeding of taiga tick larvae (IA = 5.3; IO = 60 %) compared to *M. rufocanus* (IA = 2.1; IO = 35.3 %), despite the greater abundance of the latter. Only grey-sided vole served as feeding hosts for taiga tick nymphs, with IA = 0.12 ticks per specimen and IO = 11.8 %. Ixodid ticks of any other species have not been found on rodents.

Diversity and prevalence of tick-borne infections among ixodid ticks

The prevalence of tick-borne infections in ticks

The only female of *H. concinna* was not infected by any of the pathogens studied. The agents of the North Asian tick typhus *R. sibirica* and the Far-Eastern spotted fever *R. heilongjiangensis* have not been observed in ticks or in tissue samples from rodents, and therefore these pathogens are not discussed further.

In taiga ticks collected per flag from vegetation, the most common pathogen was *B. burgdorferi* s. l., which was detected in 39.6 % *of* samples. Second abundant pathogen appeared to be *A. phagocytophilum* (16.6 %), followed by *B. miyamotoi* (8.3 %). The proportion of *Ehrlichia* sp. was 2 %. No tick-borne encephalitis virus was detected in ticks (Table 1).

The quantitative borrelia load was estimated for eight specimens of *I. persulcatus* infected with *B. burgdorferi* s. l. and for two specimens infected with *B. miyamotoi.* The mean concentration of *B. miyamotoi* was 2.5 ± 5.1 with a maximum of 2.9 log10 genome copies per tick. The concentration of *B. burgdorferi* s. l. varied from 1.1 to 3.8 and averaged 2.7 ± 0.7 log10 genome copies per tick. It can be assumed that the load of *B. burgdorferi* s. l. per tick is slightly higher than *B. miyamotoi*, although the differences are not statistically significant.

Co-infection of taiga ticks with two or more pathogens

In more than 90 % of cases, ticks were infected with only one of the tested microorganisms, but 3 (6.2 %) ticks were simultaneously infected with *B. burgdorferi* s. l. and *A. phagocytophilum*. No statistically significant associations between these infections was revealed. No other combinations of pathogens were revealed in the studied sample of taiga ticks.

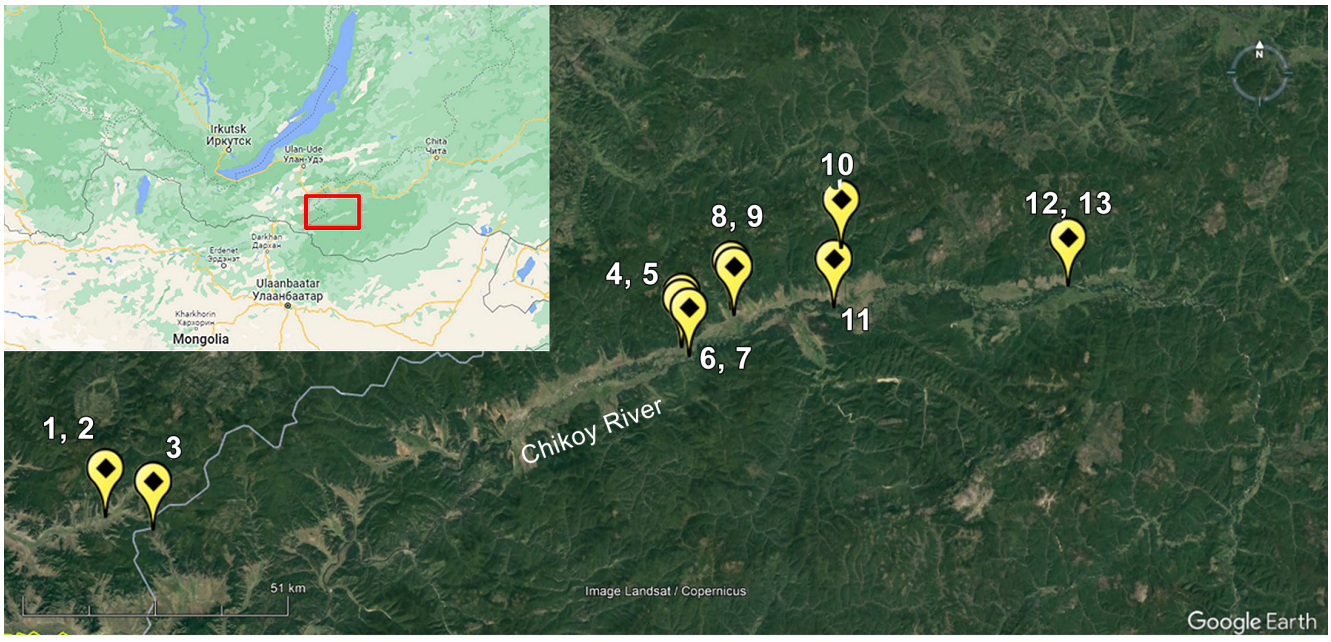

FIG. 1.

Survey area and localization of sampling sites. Mapping of tick and rodent capture sites and image creation were performed using GoogleEarthPro 7.3 software. Survey points: **1, 2** *– Republic of Buryatia, Kyakhtinsky District, vicinity of Ulady village;* **3** *– border of Buryatia and Trans-Baikal Territory;* **4–7** *– Trans-Baikal Territory, Krasnochikoysky District, Fomichevo village;* **8** *– Trans-Baikal Territory, Krasnochikoyskiy District, Zakharovo village;* **9–11** *– Trans-Baikal Territory, Krasnochikoyskiy District, Shimbylik village;* **12, 13** *– Trans-Baikal Territory, Krasnochikoyskiy District, Steklozavod village*

Diversity and prevalence of tick-borne infections among small mammalian ixodid tick feeders

All tick-borne pathogens found in taiga ticks were detected in small mammals, and two rodents were infected with TBEV – an adult female grey-sided vole and a juvenile male East Asian wood mouse – were also revealed. A. peninsulae was the most infected – about 53 % of animals were infected with at least one of the studied microorganisms. Among five specimens of *M. oeconomus*, two were infected with tick-borne pathogens. The infestation of rodents of different species is summarized in Table 2.

Mammalian infestation with tick-borne encephalitis virus

In contrast to taiga ticks, TBEV was observed in the tissues of 5 % of vertebrate hosts. In one *A. peninsulae* specimen, viral RNA was revealed in brain, and in one *M. rufocanus* specimen, systemic infection was observed with the presence of viral RNA in the brain, spleen, kidney and lungs. The prevalence of infections in of TBEV infection was comparable in both species comprising 7 % and 6 %, respectively.

Mammalian infestation and patterns of infection with B. burgdorferi s. l.

The agents of Lyme disease were the most common pathogens among vertebrate hosts and, in average, were observed in 39 % of animals, which is almost identical to the infection rate among taiga ticks. *B. burgdorferi* s. l. DNA was found in all examined mammalian species with nearly the same prevalence of 39–40 %. The exception was *M. rufocanus*, which had 35 % infection rate, although the difference was statistically insignificant.

In small mammals, *B. burgdorferi* s. l. DNA was detected in all organs examined (Table 3), although with different frequencies. In kidney tissues borreliae were found rare and only among numerous species of *A. peninsulae* and *M. rufocanus*. Most often (in almost all infected animals) the *B. burgdorferi* s.l. infection was observed in lung tissues. Among the low abundant species of *M. oeconomus* and *M. rutilus*, all infected animals

TABLE 1

THE PREVALENCE OF TICK-BORNE PATHOGENS AMONG *I. PERSULCATUS* TICKS IN THE ECOSYSTEMS OF THE CHIKOY RIVER VALLEY

	Specimens studied	Of those infected, *n*, % (95% CI)					Not infected, % (95% CI)
		TBEV	B. b. s. l.	B. m.	A. ph.	E. sp.	
I. persulcatus, females	30	0	12 40 (22; 58)	2 7 (0; 16)	4 13 (1; 25)	1 3 (0; 10)	12 40 (22; 58)
I. persulcatus, males	18	0	7 39 (16; 61)	2 11 (0; 26)	4 22 (3; 41)	0	7 39 (16; 61)
Total	48	0	19 40 (26; 53)	4 8 (1 ;16)	8 17 (6; 27)	1 2 (0; 6)	19 40 (26; 53)

Note. TBEV – tick-borne encephalitis virus; B. b. s. l. – *B. burgdorferi* sensu lato; B. m. – *B. miyamotoi*; A. ph. – *Anaplasma phagocytophilum*; E. sp. – *Ehrlichia* sp.

TABLE 2

THE PREVALENCE OF TICK-BORNE PATHOGENS AMONG SMALL MAMMALS IN THE ECOSYSTEMS OF THE CHIKOY RIVER VALLEY

Type	Specimens studied	Of those infected, *n*, % (95% CI)					Not infected, % (95% CI)
		TBEV	B. b. s. l.	B. m.	A. ph.	E. sp.	
Apodemus peninsulae	15	1 7 (0; 19)	6 40 (15; 65)	4 27 (4; 49)	1 7 (0; 19)	5 33 (9; 57)	7 47 (21; 72)
Microtus oeconomus	5	0	2 40 (0; 83)	0	1 20 (0; 55)	1 20 (0; 55)	3 60 (17; 103)
Myodes rufocanus	17	1 6 (0; 17)	6 35 (13; 58)	2 12 (0; 27)	3 18 (0; 36)	1 6 (0; 17)	9 53 (29; 77)
Myodes rutilus	1	0	1	0	1	0	0
Total	38	2 5 (0; 12)	15 39 (24; 55)	6 16 (4; 27)	6 16 (4; 27)	7 18 (6; 31)	19 50 (34; 66)

Note. TBEV – tick-borne encephalitis virus; B. b. s. l. – *B. burgdorferi* sensu lato; B. m. – *B. miyamotoi*; A. ph. – *Anaplasma phagocytophilum*; E. sp. – *Ehrlichia* sp.

had signs of systemic *B. burgdorferi* s. l. infection involving at least three organs, i.e., brain, spleen and lungs. Among *A. peninsulae*, the systemic infection was observed in three specimens with involvement of two or more organs, and in three specimens DNA of *B. burgdorferi* s. l. was detected only in lungs. In *M. rufocanus* a similar pattern was observed: three specimens exhibited systemic infection involving 3 or 4 organs, and in three cases borreliae were detected only in lungs (two specimens) or brain (one specimen).

The quantitative load of *B. burgdorferi* s. l. was possible to be determined for three specimens of *M. rufocanus* and one each of *A. peninsulae*, *M. oeconomus* and *M. rutilus* (Table 4). The attempts to quantify the spirochete concentrations in brain tissues were not successful.

Trace amounts of *B. burgdorferi* s. l. DNA were detected in the kidneys of only one of *M. rufocanus*. In spleen and lung tissues, *B. burgdorferi* s. l. DNA concentrations were comparable for most samples and were on the order of 10 genomic copies per 10 mg of tissue. In tissues of a single specimen of the *M. oeconomus* the concentration of *B. burgdorferi* s. l. DNA was 3.5 log10 genomic copies, which is about 1000 times higher than similar parameters of *M. rufocanus* and *A. peninsulae* (Table 4).

Infection and features of mammalian infection with B. miyamotoi

The agent of tick-borne relapsing fever *B. miyamotoi*, has only been found among abundant rodent species. The prevalence of infection in *A. peninsulae* was more than twice higher than in *M. rufocanus* (27 % and 12 %, respectively). In rodent organism, *B. miyamotoi* infection was more disseminated than *B. burgdorferi* s. l. (Table 5) and affected 2 or more organs in all cases, with the exception of one specimen of *A. peninsulae*, in which these spirochetes were found only in spleen.

The quantitative load of *B. miyamotoi* was determined for three infected specimens of *A. peninsulae* and two *M. rufocanus* (Table 6). Overall, the concentration of *B. miyamotoi* in mammalian tissues was about 10-fold higher than that of *B. burgdorferi* s. l. and ranged from 50 to about 10,000 genome copies per 10 mg

TABLE 3

OCCURRENCE OF *B. BURGDORFERI* S. L. IN ORGANS OF SMALL MAMMALS

Mammalian species	*B. burgdorferi* s. l. infected, specimens	Organs in which *B. burgdorferi* s. l. DNA was revealed, *n* (%)			
		brain	spleen	kidneys	lungs
A. peninsulae	6	1 (16.7)	2 (33,3)	1 (16.7)	6 (100)
M. oeconomus	2	2 (100)	2 (100)	0 (0)	2 (100)
M. rufocanus	6	3 (50)	3 (50)	2 (33.3)	5 (83.3)
M. rutilus	1	1 (100)	1 (100)	0 (0)	1 (100)

TABLE 4

MEAN BACTERIAL LOAD OF *B. BURGDORFERI* S. L. IN ORGANS OF SMALL MAMMALS

Mammalian species	*B. burgdorferi* s. l. concentration in tissues (lg genome copies/10 mg)		
	spleen	kidneys	lungs
A. peninsulae	1	0	0.8
M. oeconomus	3.5	0	3.3
M. rufocanus	0	0.1	0.9
M. rutilus	0.6	0	1.4

of tissue. In *A. peninsulae*, the quantitative load of spirochetes in different organs was approximately equal, whereas in *M. rufocanus*, spleen tissues were prominent, in which the DNA concentration of *B. miyamotoi* was 2 orders of magnitude higher than in other organs (Table 6).

Prevalence and patterns of infection with *A. phagocytophilum* in rodents

Overall, the causative agent of human granulocytic anaplasmosis (HGA), *A. phagocytophilum*, was found in 16 % of mammals but the incidence of infection with this pathogen varied between species. Thus, *M. rufocanus* and *M. oeconomus* were characterized by infection rates close to the mean value, but among *A. peninsulae* the prevalence of infection was three times lower (18–20 % vs. 7 %, respectively; Table 2).

In most cases, *A. phagocytophilum* infection resulted in a systemic spread of the pathogen involving all organs examined, except the kidneys (Table 7). The only exception was an infected specimen of *A. peninsulae*, in which HGA agent DNA of the human granulocytic anaplasmosis pathogen was detected only in the spleen (Table 7).

Prevalence and patterns of infection with Ehrlichia sp. in rodents

Ehrlichia infection was most common of *A. peninsulae*, in which these microorganisms were observed in 33 % of cases. Among other mammalian species, these bacteria were found in single specimens (Table 2).

TABLE 5

OCCURRENCE OF *B. MIYAMOTOI* IN ORGANS OF SMALL MAMMALS

Mammalian species	*B. miyamotoi* infected, specimens	Organs in which *B. miyamotoi* DNA was revealed, *n* (%)			
		brain	spleen	kidneys	lungs
A. peninsulae	4	2 (50)	4 (100)	0 (0)	3 (75)
M. rufocanus	2	0 (0)	1 (50)	1 (50)	2 (100)

TABLE 6

MEAN BACTERIAL LOAD OF *B. MIYAMOTOI* IN ORGANS OF SMALL MAMMALS

Mammalian species	*B. miyamotoi* concentration in tissues, lg genome copies/10 mg			
	brain	spleen	kidneys	lungs
A. peninsulae	2	2.7	0	2.3
M. rufocanus	0	4.1	1.4	2.2

TABLE 7

OCCURRENCE OF *A. PHAGOCYTOPHILUM* IN ORGANS OF SMALL MAMMALS

Mammalian species	Infected with *A. phagocytophilum*, specimens	Organs in which *A. phagocytophilum* DNA was revealed, *n* (%)		
		brain	spleen	lungs
A. peninsulae	1	0	1 (100)	0
M. oeconomus	1	1 (100)	1 (100)	1 (100)
M. rufocanus	3	2 (66.7)	3 (100)	3 (100)
M. rutilus	1	1 (100)	1 (100)	1 (100)

In contrast to all other pathogens studied, *Ehrlichia* sp. infection was always systemic, and in *A. peninsulae*, ehrlichiae DNA was detected in all organs examined (Table 8).

Co-infection of small mammals with two or more pathogens

Co-infection with two or more pathogens appeared to be widespread among vertebrate hosts of tick-borne infections. The only *M. rufocanus* specimen studied was simultaneously infected with two pathogens, i. e. *B. burgdorferi* s. l. and *A. phagocytophilum*. Of the two infected *M. oeconomus*, one was infected with three bacteria species, i. e. *B. burgdorferi* s. l., *A. phagocytophilum* and *Ehrlichia* sp. The pattern of infection with tick-borne pathogens among multiple mammalian species is summarized in Table 9.

Korean field mice *A. peninsulae* were co-infected more often – about 40 % of animals were simultaneously infected with two or more pathogens, with mono-infections only be-

TABLE 8

DISTRIBUTION OF *EHRLICHIA* SP. IN VARIOUS ORGANS OF SMALL MAMMALS

Mammalian species	*Ehrlichia* sp. infected, specimens	Organs in which *Ehrlichia* sp. DNA was revealed, n (%)			
		brain	spleen	kidneys	lungs
A. peninsulae	5	4 (80)	5 (100)	1 (20)	5 (100)
M. oeconomus	1	1 (100)	1 (100)	0	1 (100)
M. rufocanus	1	1 (100)	1 (100)	0	1 (100)

TABLE 9

CO-INFECTION PATTERNS OF TICK-BORNE PATHOGENS IN SMALL MAMMALS

Pathogens	Mono-infection, *n* (%)	Co-infection, *n* (%)	Co-infection with (%)				
			B. b. s. l.*	B. m.	A. ph.	E. sp.	more than 2 pathogens
			A. peninsulae (*n* = 15)				
TBEV	0	1 (6.7)	0	0	0	0	1 (6.7)
B. b. s. l.	0	6 (40)	–	1 (6.7)	1 (6.7)	2 (13.3)	2 (13.3)
B. m.	1 (6.7)	3 (20)	1 (6.7)	–	0	0	2 (13.3)
A. ph.	0	1 (6.7)	1 (6.7)	0	–	0	0
E. sp.	1 (6.7)	4 (26.7)	2 (13.3)	0	0	–	2 (13.3)
Total	2 (13.3)	6 (40)	4 (26.7)	1 (6.7)	1 (6.7)	2 (13.3)	–
			M. rufocanus (*n* = 17)				
TBEV	1 (5.9)	0	0	0	0	0	0
B. b. s. l.	2 (11.8)	4 (23.5)	–	1 (5.9)	2 (11.8)	0	1 (5.9)
B. m.	1 (5.9)	1 (5.9)	1 (5.9)	–	0	0	0
A. ph.	0	3 (17.6)	2 (11.8)	0	–	0	1 (5.9)
E. sp.	0	1 (5.9)	0	0	0	–	1 (5.9)
Total	4 (23.5)	4 (23.5)	3 (17.6)	1 (5.9)	2 (11.8)	0	–

Note. TBEV – tick-borne encephalitis virus; B. b. s. l. – *B. burgdorferi* sensu lato; B. m. – *B. miyamotoi*; A. ph. – *Anaplasma phagocytophilum*; E. sp. – *Ehrlichia* sp.

ing observed for *B. miyamotoi* and *Ehrlichia* sp. The only case of TBEV infection in this rodent species was observed only in co-infection with *B. burgdorferi* s. l., *B. miyamotoi* and *Ehrlichia* sp. Among the bacteria, *B. burgdorferi* s. l. were observed with approximately equal frequency in all co-infections; *B. miyamotoi* was observed either in multiple co-infections or in combination with *B. burgdorferi* s. l., the only case of *A. peninsulae* infection with anaplasmas was in combination with *B. burgdorferi* s. l., whereas the more common ehrlichiae occurred in about equal numbers, both as mono-infections and in various combinations with other microorganisms.

Among *M. rufocanus*, approximately 25 % of animals were infected with one pathogen and the same number – with two or more pathogens. Mono-infections have been reported for TBEV, *B. burgdorferi* s. l. and *B. miyamotoi*. Among the co-infections, the most notable were *B. burgdorferi* s. l., which occurred in all available combinations except TBEV. For the other bacteria, co-infections in this rodent species were not common and were limited to single cases of multiple infections or co-infections with *B. burgdorferi* s. l.

CONCLUSION

In the Krasnochikoysky district of the Trans-Baikal Territory within the Chikoy River valley, a stable circulation of at least five causative agents of tick-borne infections has been revealed for the first time: tick-borne encephalitis virus, *B. burgdorferi* s. l., *B. miyamotoi, A. phagocytophilum* and *Ehrlichia* sp. It has been shown that these pathogens not only infect tick vectors, but are also spread among natural vertebrate hosts – small mammals. *I. persulcatus* ticks are the most relevant vectors of tick-borne infections in the surveyed area, while the most important vertebrate hosts and tick feeders are *M. rufocanus* and *A. peninsulae*.

No cases of infection of taiga ticks and rodents with causative agents of tick-borne rickettsiosis of northern Asia *R. sibirica* and Far Eastern tick-borne rickettsiosis *R. heilongjiangensis* were revealed. However, the existence of natural foci of these diseases in the ecosystems of the Chikoy River catchment cannot be excluded. A limited sample size of ticks and vertebrate hosts, on the one hand, does not allow us to assert the completeness of the description of the ixodid tick fauna of the Chikoy River valley, as well as the biodiversity of pathogens infecting them. In contrast, the discovery of the *H. concinna* tick (which is the competent invertebrate host of *R. heilongjiangensis*), also suggests that the species diversity of both tick vectors and tick-borne pathogens in the Krasnochikoisky district may be even wider than established in the present study.

Summarising the results obtained, we can note a high risk of infection with tick-borne encephalitis, Lyme disease, tick-borne relapsing fever caused by *B. miyamotoi*, HGA and HME in the Krasnochikoisky district of the Trans-Baikal Territory, which is part of the Baikal Natural Area, including the most attractive areas for residence and recreation of the local population, Russian and foreign tourists. This evaluation is supported by the widespread distribution of epidemically significant vectors of tick-borne infections – *I. persulcatus* ticks, the presence of numerous populations of competent vertebrates – hosts of infections and tick feeders, high prevalence of infections in of ticks and small mammals, as well as a significant proportion of animals simultaneously infected with two or more pathogens. All this indicates the need to further improve the surveillance of tick-borne infections with the involvement of all the possibilities of modern science for both short-term and medium- and long-term forecasting of the epidemiological situation.

Conflict of interest

The authors of this article declare no conflicts of interest.

Compliance with the principles of biomedical ethics

The study was designed and performed in compliance with the ethical principles required by the World Medical Association Declaration of Helsinki. The study was performed with the approval of the Biomedical Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 2 from February 18, 2020).

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Information about the authors

Ekaterina K. Lagunova – Laboratory Assistant at the Laboratory of Vector-Borne Infections, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: lagunovakatya1994@yandex.ru, https://orcid.org/0009-0007-3688-023X

Maxim A. Khasnatinov – Dr. Sc. (Biol.), Leading Research Officer at the Laboratory of Vector-Borne Infections, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: khasnatinov@yandex.ru, https://orcid.org/0000-0002-8441-3640

Galina A. Danchinova – Dr. Sc. (Biol.), Head of the Laboratory of Vector-Borne Infections, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: dan-chin@yandex.ru, https://orcid.org/0000-0002-6705-3970

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MORPHOLOGY, PHYSIOLOGY AND PATHOPHYSIOLOGY

PHARMACOLOGICAL BLOCKADE OF CANNABINOID TYPE II RECEPTORS AND MESENCHYMAL STEM CELL TRANSPLANTATION IN A MODEL OF PERIPHERAL NEUROPATHIC PAIN

Yerofeyeva A.-M.V. [1],
Pinchuk S.V. [2],
Rjabceva S.N. [1],
Molchanova A.Yu. [1]

[1] Institute of Physiology, National Academy of Sciences of Belarus (Akademicheskaya str. 28, Minsk 220072, Republic of Belarus)
[2] Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus (Akademicheskaya str. 28, Minsk 220072, Republic of Belarus)

Corresponding author:
Anna-Maria V. Yerofeyeva,
e-mail: amyerofeyeva@zoho.eu

ABSTRACT

The aim of the study. *To evaluate the anti-nociceptive and reparative effects of adipose-derived mesenchymal stem cells (ADMSCs) under the pharmacological blockade of cannabinoid CB_2 receptors in a model of peripheral neuropathic pain.*
Material and methods. *In 40 male Wistar rats, modeling of peripheral neuropathy (NP) was performed by excising a sciatic nerve. On day 7 of the study, ADMSCs (1×10^6 cells/kg) were transplanted into the area of sciatic nerve injury without additional influences or after administration of the CB_2 receptor antagonist AM630, as well as after incubation with AM630. Within 90 days, nociceptive sensitivity was studied, as well as a detailed analysis of gait using CatWalk XT (Noldus, Netherlands). On day 21 and day 90, histostructure of the distal segment of the sciatic nerve was assessed.*
Results. *Pharmacological blockade of CB_2 receptors both on the ADMSCs and in the soft tissues surrounding the site of sciatic nerve injury led to a decrease in withdrawal threshold and withdrawal latency from day 28 of the study compared with the group of rats with NP and transplantation of ADMSCs only. Local injection of AM630 before transplantation of ADMSCs contributed to the development of NP-induced gait disturbances and increase of the number of damaged nerve fibers in the distal segment of sciatic nerve. Transplantation of ADMSCs pretreated with AM630 did not significantly affect the rate of recovery of gait parameters, and decreased the number of damaged nerve fibers by day 90 of study.*
Conclusion. *Blockade of CB_2 receptors, both on the membranes of MSCs and in the area of damage to the peripheral nerve, has a negative effect on the development of the anti-nociceptive and reparative effects of MSCs.*

Key words: *mesenchymal stem cells, neuropathic pain, sciatic nerve, cannabinoid receptors, pharmacological blockade of CB_2 receptors*

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ФАРМАКОЛОГИЧЕСКАЯ БЛОКАДА КАННАБИНОИДНЫХ РЕЦЕПТОРОВ II ТИПА ПРИ ТРАНСПЛАНТАЦИИ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК В МОДЕЛИ ПЕРИФЕРИЧЕСКОЙ НЕЙРОПАТИЧЕСКОЙ БОЛИ

Ерофеева А.-М.В. [1],
Пинчук С.В. [2],
Рябцева С.Н. [1],
Молчанова А.Ю. [1]

[1] ГНУ «Институт физиологии НАН Беларуси»
(220072, г. Минск, ул. Академическая, 28, Республика Беларусь)
[2] ГНУ «Институт биофизики и клеточной инженерии НАН Беларуси»
(220072, г. Минск, ул. Академическая, 27, Республика Беларусь)

Автор, ответственный за переписку:
Ерофеева Анна-Мария Вадимовна,
e-mail: amyerofeyeva@zoho.eu

РЕЗЮМЕ

Цель исследования. *Оценить антиноцицептивный и репаративный эффекты мезенхимальных стволовых клеток жировой ткани (МСК ЖТ) на фоне фармакологической блокады каннабиноидных рецепторов CB_2 в модели периферической нейропатической боли.*

Материал и методы. *У 40 крыс-самцов Wistar осуществили моделирование периферической нейропатии (НП) путём иссечения участка седалищного нерва. На 7-е сутки исследования проведена трансплантация МСК ЖТ (1×10^6 клеток/кг) в область повреждения седалищного нерва без дополнительных воздействий, а также после локального введения антагониста CB_2-рецептора AM630 и предварительной инкубации с AM630. В течение 90 суток регистрировали ноцицептивную чувствительность и анализировали походку крыс с помощью CatWalk XT (Noldus, Нидерланды). На 21-е и 90-е сутки проведена оценка гистоструктуры дистального сегмента седалищного нерва после аксотомии.*

Результаты. *Фармакологическая блокада CB_2-рецепторов как на мембранах МСК ЖТ, так и в мягких тканях, окружающих место повреждения седалищного нерва, приводила к снижению порога и латентного периода ноцицептивной реакции с 28-х суток исследования по сравнению с группой крыс с НП и группой животных после трансплантации только МСК ЖТ. После локального введения AM630 перед трансплантацией МСК ЖТ отмечены ухудшение параметров походки, вызванные НП, и увеличение доли повреждённых нервных волокон в дистальном сегменте седалищного нерва. Трансплантация МСК, преинкубированных с AM630, не оказывала существенного влияния на скорость восстановления параметров походки, к 90-м суткам исследования сопровождалась снижением числа повреждённых нервных волокон.*

Заключение. *Блокада CB_2-рецепторов как на мембранах МСК, так в зоне повреждения периферического нерва сопровождается снижением антиноцицептивного эффекта МСК при их локальной трансплантации и подавляет репаративное действие МСК.*

Ключевые слова: *мезенхимальные стволовые клетки, нейропатическая боль, седалищный нерв, каннабиноидные рецепторы, фармакологическая блокада CB_2-рецепторов*

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INTRODUCTION

Chronic pain syndromes of neurogenic origin, associated with damage or dysfunction of peripheral parts of the somatosensory nervous system [1], occur in 7–20 % of the European population [2] and in 30–50 % of the world population [3]. Cell therapy using adipose-derived mesenchymal stem cells (ADMSC) appears promising for peripheral nerve injuries and related pain syndromes [4, 5]; their antinociceptive effect with local transplantation has been evidenced in models of peripheral neuropathy of various etiologies [6–11]. The ability of MSC to alleviate neuropathic pain is currently associated mainly with the suppression of local inflammatory response by secretion of a number of paracrine factors [5], but the mechanisms of activation of these processes are not fully disclosed.

Cannabinoid CB_2 receptors are involved in modulating the transduction, transmission and processing of nociceptive signals at both the peripheral and central levels of the somatosensory nervous system [12] and serve as a target for pain management. MSCs are able to produce CB_2 receptor ligands [13], which may be one of the mechanisms of their analgesic action. Conversely, CB_2 receptors are present in MSCs and are involved in sustaining their viability and metabolic activity [13–16], indicating a possible interaction between MSCs and endogenous cannabinoids in the transplantation area. It is essential to study the changes of MSC effects under the conditions of blockade of these receptors in order to understand the role of CB_2-receptors in analgesic and reparative action of MSC at local injection into the area of peripheral nerve injury.

THE AIM

Assessment of antinociceptive and reparative effects of adipose-derived mesenchymal stem cells against pharmacological blockade of cannabinoid CB_2 receptors in a model of peripheral neuropathic pain.

MATERIALS AND METHODS

The study was conducted on 40 male Wistar rats weighing 180–200 g. The animals were kept in the vivarium of the Institute of Physiology, National Academy of Sciences of Belarus with free access to water and food and 12/12 h day/night cycle. Animals were divided into 4 groups by simple randomization method (using random number table), in each group $n = 10$:

1) rats with peripheral neuropathy (NP) model without treatment (NP group);

2) rats with NP model and transplantation of allogeneic ADMSC to the area of sciatic nerve injury (NP + ADMSC group);

3) rats with NP model and ADMSC transplantation under pharmacological blockade of CB_2 receptors in soft tissues of the sciatic nerve injury area (NP + AM630 + ADMSC group);

4) rats with NP model and ADMSC transplantation, which underwent pharmacological blockade of CB_2 receptors when pre-incubated with selective antagonist (NP + pre-AM630-ADMSC group).

All manipulations involving experimental animals were undertaken in compliance with the principles of bioethics as set out in the European Convention for the Protection of Vertebrates Animals used for Experimental and other Scientific Purposes. The study minutes were approved by the Bioethics Commission of the Institute of Physiology, National Academy of Sciences of Belarus (Minutes No. 1 dated February 02, 2023).

Surgical manipulations. NP was modelled on the left hind limb of rats by axotomy of a 0.5 cm section of the sciatic nerve according to the previously described method [8]. The surgery was performed under general anaesthesia induced by intravenous injection of sodium thiopental (JSC 'Sintez', Russia) at a dose of 20 mg/kg with local anaesthesia (lidocaine hydrochloride (JSC 'Borisov Medical Preparations Plant', Republic of Belarus), 0.1 ml intramuscularly).

ADMSC transplantation. On day 7 after NP modelling, experimental groups were injected with ADMSCs in the amount of 1×10^6 cells/kg. ADMSCs isolated from visceral fat of intact rats were cultured in a CO_2 incubator (37 °C; 5% CO_2) until the 3rd passage according to the previously described method [17]. Using flow cytofluorimeter FACSCanto II (Becton Dickinson, USA) the phenotype of ADMSC was analysed by the presence of characteristic markers CD29, CD44 and CD90 and absence of hematopoietic marker CD45. Cell suspension in phosphate-buffered saline (PBS, phosphate-buffered saline) buffer (pH = 7.2; Sigma-Aldrich, Germany) was injected intramuscularly with an insulin syringe with an integrated 30 G needle in four injections around the area of surgical excision of the nerve site according to an imaginary dial pattern at 3, 6, 9, and 12 hours.

CB_2 receptor pharmacological blockade. To achieve blockade of CB_2 receptors on ADMSC's membranes, cells were incubated with the selective antagonist AM630 (Sigma-Aldrich, Germany) for 24 h (2 μM). Pharmacological blockade of CB_2-receptors in soft tissues of the sciatic nerve injury area was performed by intramuscular injection of AM630 (100 μg/kg) 15 min before ADMSC transplantation. The AM630 antagonist was diluted in a solvent consisting of sterile PBS buffer (pH = 7.4; Sigma-Aldrich, Germany) and 0.2 % dimethyl sulfoxide (NeoFroxx GmbH, Germany).

An assessment of nociceptive sensitivity. The Randall – Selitto algesimeter (Panlab, Spain) was used to determine nociceptive sensitivity to a mechanical stimulus (mechanical withdrawal threshold (MWT)), and Hot Plate' algesimeter (Panlab, Spain) was used to determine nociceptive sensitivity to a thermal stimulus (thermal withdrawal latency (TWL)) [18]. Measurements were performed three times with an interval of 5–7 min. Noci-

ceptive sensitivity was assessed on days 0, 7, 14, 21, 28, 60 and 90 of the study.

Analysis of gait parameters. Detailed analysis of gait was performed using the software and hardware complex CatWalk XT 10.6 (Noldus, Netherlands). The system enables qualitative and quantitative assessment of gait parameters during the animal's free movement on a glass platform. The intensity of green light at the point of contact between the paws and the surface of the glass podium illuminated with green LED lighting was recorded with a high-speed video camera with a wide-angle lens Gevicam GP-3360 (GEViCAM Inc., USA) located under the corridor. Paw prints were analysed and gait parameters were calculated using the software of this hardware-software complex. Prior to the study, the animals were adapted to the device and run recording conditions. Runs of each animal were recorded in a dark, ventilated room 3 times each with a maximum step variability of 60 % and a run time of no more than 5.00 s. The analysis included static and dynamic parameters, which reflect the degree of tonic pain sensations in this model and also indirectly demonstrate the functional state of the sciatic nerve. Dynamic parameters included:

Stand Time – duration of the paw stand phase on the ground;

Swing Time – duration of the paw swing phase in the air;

Duty Cycle – working cycle of the paw, the ratio of the duration of the paw stand phase to the duration of the full step cycle.

Static gait parameters:

Print Length – the length of the print;

Print Width – the width of the print;

Print Area – the area of the print;

Max contact area – the area of the print at the most intensive contact of the paw with the platform;

Max intensity – the maximum intensity of the paw print;

Mean intensity – the average intensity of the paw print;

Sciatic functional index (SFI).

These parameters were selected based on previous studies [6–7, 19]. To exclude the effects of run speed and body weight of animals influencing gait parameters, data were calculated as a percentage of the contralateral hind paw, except for SFI.

Assessment of the sciatic nerve histostructure. On days 21 and 90 of the study, a distal segment of the sciatic nerve was sampled for histological examination. The samples were fixed in 10 % neutral buffered formalin. Sections 5 μm thick were stained with hematoxylin and eosin according to standard techniques. The obtained preparations were viewed and digitized using an Optec BK 5000 light microscope with a digital camera (Optec, China) at a magnification of × 400. The percentage of normal and damaged nerve fibres was counted on transverse sections of the sciatic nerve [20]. Morphometric parameters were assessed in the field of view of a microscope at × 400 magnification (area 67072.0 μm^2) in at least five fields of view.

Statistics. Statistical processing of data was performed using Statistica 10 software (StatSoft Inc., USA). Data were checked for normality of distribution by the Shapiro – Wilk test. In normal characteristic distribution, results are presented as mean ± standard deviation (M ± SD); in an abnormal distribution, results are presented as median and quartiles (Me (Q25; Q75)). Differences in nociceptive sensitivity and gait parameters were assessed by repeated measures analysis of variance with posterior comparisons using the least significant difference method. Morphometric data were compared by the Kruskal – Wallis criterion followed by a posteriori comparisons. The conclusion about statistical significance of differences was made at $p < 0.05$.

RESULTS

Alterations in nociceptive sensitivity. On day 7 after NP modelling, the development of mechanical and thermal hyperalgesia was observed. This was evidenced by a 35.5 % decrease in ipsilateral limb MWT (from 136.0 ± 1.9 to 87.7 ± 2.0 g) and 34.3 % decrease in TWL (from 18.1 ± 0.6 to 11.9 ± 0.4 s) relative to baseline values ($p < 0.001$) (Fig. 1a, b). There was no tendency for MWT and TWL to recover to their original values over the course of the study. The MWT of the contralateral healthy limb did not change statistically significantly throughout the study ($p > 0.05$ compared to day 0; Fig. 1a).

A single intramuscular injection of ADMSC into the area of sciatic nerve injury resulted in a 32.3 % increase in the MWT of the ipsilateral extremity by day 14 (from 85.4 ± 2.0 to 113.0 ± 1.9 g; $p < 0.001$ by day 7; Fig. 1a), TWL by 17.1 % (from 11.7 ± 0.5 to 13.7 ± 0.5 s; $p < 0.001$ by day 7; Fig. 1b). By day 21 of the study, the MWT of the ipsilateral limb had already increased to 129.4 ± 2.0 g ($p < 0.001$ by day 7; $p > 0.05$ by day 0; Fig. 1a) and the TWL to 16.2 ± 0.5 s (38.5 % higher; $p < 0.001$ by day 7; $p > 0.05$ by day 0; Fig. 1b). Further up to and including day 90, MWT and TWL were not statistically significantly different from baseline values ($p > 0.05$; Fig. 1a, b). Administration of ADMSC to rats with NP also statistically significantly increased their MWT and MWT when compared to untreated animals already on day 14 day of the study: MWT was higher by 26.2 % ($p < 0.001$), and TWL – by 20.2 % ($p < 0.001$). From day 21 onwards, the increase in MWT relative to the untreated NP group was 53.5 % ($p < 0.001$), TWL was 54.3 % ($p < 0.001$).

Antagonist AM630 administration at a dose of 100 μg/kg to the area of nerve injury in rats with NP on day 7 of the study did not lead to statistically significant changes in MWT of the ipsilateral extremity, as well as TWL after 15 min (Table 1).

ADMSC injection 15 min after pharmacological blockade of CB_2 receptors in the area of sciatic nerve injury resulted in a 21.9 % increase in MWT on day 14 of the experiment relative to the values on day 7

(from 94.5 ± 2.1 to 115.2 ± 2.6 g; $p < 0.001$). And these values were not statistically significantly different from the NP + ADMSC group ($p > 0.05$), but were 18.7 % higher than in the NP group without treatment ($p < 0.001$). By day 21, MWT tended to decrease compared to the NP + ADMSC group and tended to increase by 33.3 % compared to the NP group without treatment ($p < 0.001$). On day 28, MWT increased to 120.4 ± 4.1 g, which was 9.3 % lower than the values of the NP + ADMSC group ($p < 0.001$) and 33.2 % higher than those of untreated rats ($p < 0.001$; Fig. 1a). Thereafter, a decreasing trend in MWT was observed (Fig. 1a). An increase in TWL by day 14 of the study by 21.5 % was observed in relation to the values obtained on day 7: from 10.7 ± 0.3 to 13.0 ± 0.3 s ($p < 0.001$; no statistically significant differences with the NP + ADMSC group ($p > 0.05$); 14.0 % higher in comparison with the PN group without treatment ($p < 0.05$) (Fig. 1b). By day 28, TWL increased to 13.1 ± 0.2 s, and the index was 22.0 % lower than in the NP + ADMSC group ($p < 0.001$). By day 90 of the study, we observed a marked decrease in TWL to 11.5 ± 0.3 s ($p > 0.05$ compared to NP without treatment; $p < 0.001$ compared to NP + WL MSCs; Fig. 1b).

ADMSC injection preincubated with AM630 increased the MWT of the ipsilateral extremity on day 14 of the study by 18.3 % relative to the values obtained on day 7 (from 95.9 ± 1.4 to 113.4 ± 2.0 g; $p < 0.001$; Fig. 1a) and TWL by 13.3 % relative to the values obtained on day 7 (from 10.5 ± 0.4 to 11.9 ± 0.3 s; $p < 0.001$). Meanwhile, MWT was not significantly different from the values in the NP + ADMSC group ($p > 0.05$) and in the NP + AM630 + ADMSC group

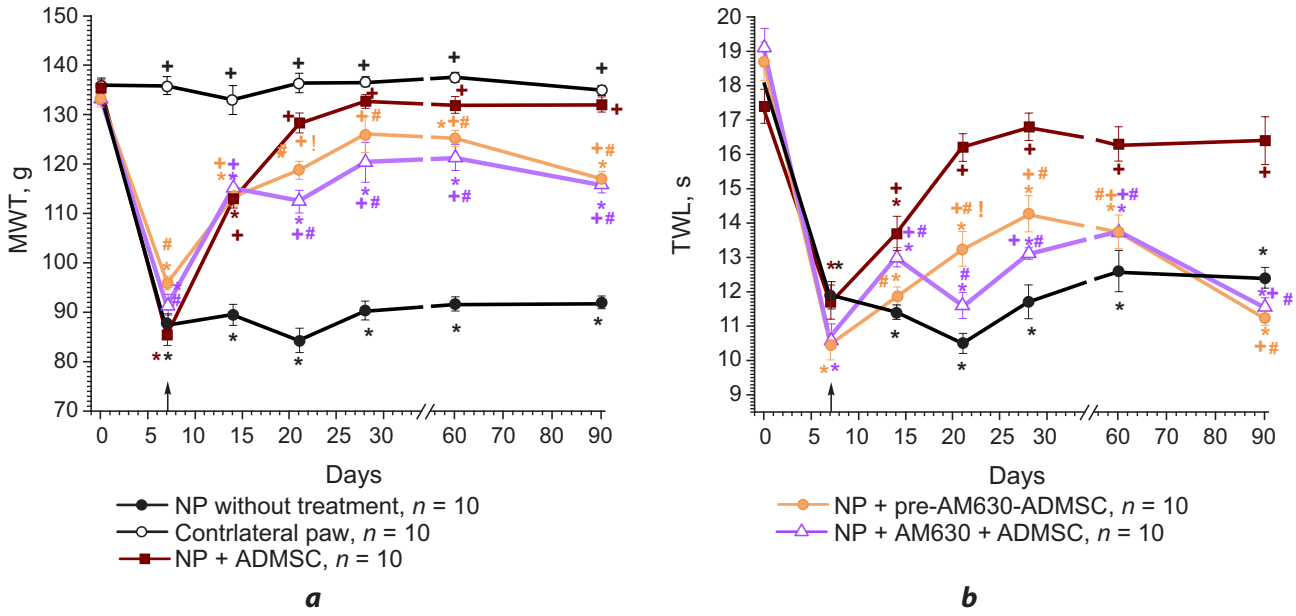


FIG. 1.

*Dynamics of nociceptive sensitivity to mechanical (**a**) and thermal (**b**) stimuli in rats with NP, ADMSC transplantation on the background of pharmacological blockade of CB_2-receptors by AM630 antagonist: arrow indicates the time of ADMSC transplantation; * – $p < 0.05$ compared to day 0; + – $p < 0.05$ compared to NP without treatment; # – $p < 0.05$ compared to NP + ADMSC; ! – $p < 0.05$ compared to NP + AM630 + ADMSC*

TABLE 1

NOCICEPTIVE SENSITIVITY PARAMETERS IN RATS WITH PN AFTER ADMINISTRATION OF AM630 ANTAGONIST TO THE SITE OF SCIATIC NERVE INJURY

Parameters	MWT (left paw), g	MWT (right paw), g	TWL, s
Day 0	133.2 ± 1.3	132.5 ± 1.0	19.1 ± 0.6
Day 7	91.2 ± 1.8*	133.9 ± 0.9	10.6 ± 0.3*
15 min after AM630 injection	94.5 ± 2.1*	131.6 ± 1.3	10.7 ± 0.3*

Note. * – $p < 0.05$ compared to day 0.

($p > 0.05$), whilst TWL was 13.1 % lower than the values of the NP + ADMSC group and was not statistically significantly different from the group with NP without treatment ($p > 0.05$). By day 21, both parameters in this group were lower than those in the rats that had only received ADMSC by 8.3 % ($p < 0.05$) and 18.5 % ($p < 0.001$), respectively, but higher than those in the NP + AM630 + ADMSC group by 5.6 % and 13.8 %, respectively ($p < 0.05$). By day 90, a decrease in MWT to 117.0 ± 1.5 g ($p < 0.001$ to NP group without treatment; $p < 0.001$ to NP + ADMSC group) and TWL to 11.3 ± 0.2 s ($p > 0.05$ to NP without treatment; $p < 0.001$ to PN + ADMSC) were observed.

By comparing both methods of pharmacological blockade of CB_2-receptors it was revealed that after transplantation of ADMSC preincubated with AM630 there was observed an increase of MWT relative to NP + AM630 + ADMSC group on day 21 of the study by 5,6 % ($p < 0.05$), and also a decrease of TWL on day 14 by 8.5 % ($p < 0.05$) with the subsequent increase on day 21 of the study by 13.8 % ($p < 0.02$). At later terms of the study no statistically significant differences in the studied indices between these groups were revealed. Overall, ADMSC transplantation following local injection of AM630 resulted in a more pronounced reduction in the antinociceptive action of ADMSC.

Dynamic gait parameters. In the untreated NP group, a reduction in the duration of the ipsilateral paw stand duration to 91.0 % ($p < 0.001$) and of the duty cycle to 95.4 % ($p < 0.001$) was observed from day 7 of the experiment, with no statistically significant differences in the swing duration ($p > 0.05$; Fig. 2c). By day 21 of the study, there was an adaptive recovery of the above parameters (Fig. 2a, b).

After ADMSC injection at a dose of 1×10^6 cells/kg into the area of sciatic nerve transection, the recovery of the stand duration (up to 98.4 %) and duty cycle (up to 101.9 %) was observed by day 14 (Fig. 2a, b),

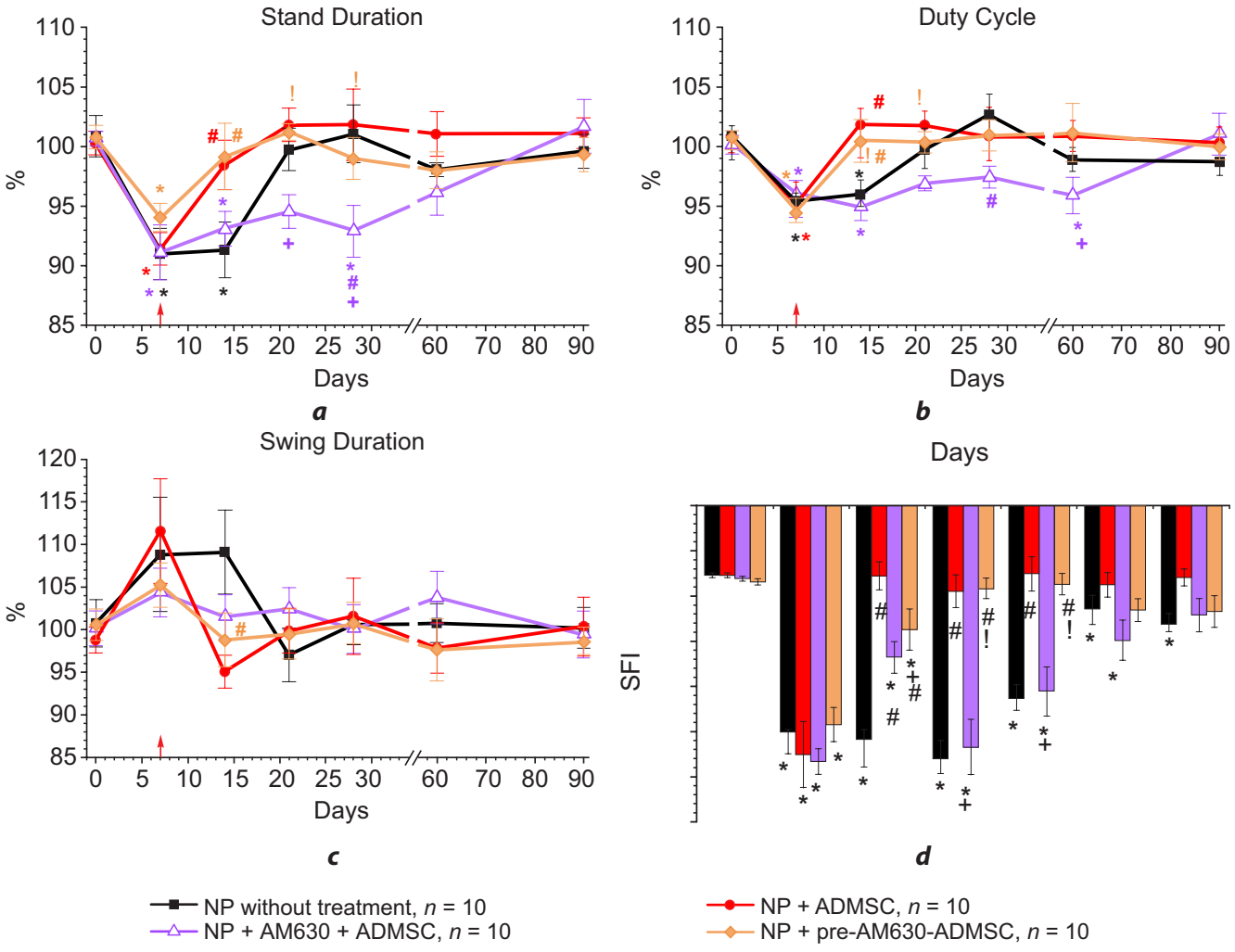


FIG. 2.

*Gait dynamic parameters (**a**–**c**) and functional sciatic index (**d**) changes in rats after NP modelling, ADMSC transplantation on the background of pharmacological blockade of CB_2 receptors by AM630 antagonist: arrow indicates the time of transplantation; * – $p < 0.05$ to the values before NP modelling; # – $p < 0.05$ to the NP group without treatment; + – $p < 0.05$ to NP + ADMSC; ! – $p < 0.05$ to the NP + AM630 + ADMSC group*

and no further changes were revealed. After ADMSC injection against the background of pharmacological blockade of CB_2-receptors in the area of sciatic nerve transection, the recovery of the stand duration was fixed by day 60 of the study (Fig. 2a), and of the duty cycle – by day 90 of the study (Fig. 2b). On day 28 of the experiment, there was a decrease in the stand duration of the ispilateral paw compared to the NP group without treatment (by 8.1 %; $p < 0.005$) and duty cycle (by 5.1 %; $p < 0.01$). Compared to the NP + ADMSC group, the stand duration of the ipsilateral paw was shorter on day 21 (7.1 %; $p < 0.05$) and day 28 (8.8 %; $p < 0.002$) of the study; the duty cycle was shorter on day 14 (6.8 %; $p < 0.005$) and day 60 (4.9 %; $p < 0.02$) of the study. Pre-AM630-MSC injection of ADMSCs resulted in restoration of the stand duration to 99.2 % by day 14 ($p > 0.05$ by day 0; $p < 0.05$ for the NP group without treatment) and of the duty cycle to 100.5 % ($p > 0.05$ by day 0; $p < 0.05$ for the NP group without treatment). No statistically significant differences in dynamic parameters from the NP + ADMSC group were revealed, and the pattern of changes in these gait parameters in these two groups was similar.

Sciatic functional index. This index in the untreated NP group decreased 2.3-fold, by 229.9 % (from –7.65 ± 0.30 to –24.99 ± 2.45; $p < 0.001$ compared to the data on day 0) by day 7 of the study (Fig. 2d). The tendency to recovery of this index was observed only from the day 60 of the study, but by day 90 of the study no recovery of SFI to the baseline level was observed ($p < 0.01$) (Fig. 2d). After ADMSC transplantation, recovery of SFI to –7.75 ± 1.57 was observed by day 14 of the study. Further, no statistically significant changes in SFI were observed throughout the study compared to the values on day 0. If AM630 was administered 15 min before ADMSC transplantation, a partial recovery of SFI was observed by day 14 of the study (to –16.75 ± 1.74; $p < 0.002$ by day 0; $p < 0.05$ to the untreated NP group). From day 21 of the study, the index decreased again to the level of the NP group without treatment (to –26.69 ± 3.05; $p < 0.001$ by day 0; $p > 0.05$ to the NP group without treatment). SFI in this group returned to baseline only by day 90 of the study (to 12.10 ± 1.85), but was not statistically significantly different from NP without treatment. Compared to the NP + ADMSC group, SFI in this group was statistically significantly decreased on day 14 ($p < 0.001$), day 21 ($p < 0.001$) and day 28 ($p < 0.001$) of the experiment. After transplantation of ADMSC preincubated with AM630, SFI was fully recovered by day 21 (to –9.16 ± 1.12; $p > 0.05$ by day 0; $p < 0.001$ to the NP group without treatment). Compared to the NP + ADMSC group, SFI in this group was lower on day 14 of the study (by 76.4 %; $p < 0.005$).

Static gait parameters. From day 28 onwards, the untreated NP group observed a decrease in the print length of the injured paw compared to day 0 to 93.6 % ($p < 0.02$; Fig. 3a), and in the print width to 96.1 % ($p < 0.001$; Fig. 3b). The print area of the ipsilateral extremity decreased to 84.5 % of the contralateral paw ($p < 0.001$; Fig. 3c), the max contact area decreased to 84.7 %, ($p < 0.005$; Fig. 3f), the max intensity decreased to 91.5 % ($p < 0.001$; Fig. 3d), and the mean intensity decreased to 98.3 % ($p < 0.01$; Fig. 3e). No tendency to recovery of static gait parameters in animals of this group was revealed up to day 90 of observation inclusive.

No statistically significant changes in static parameters were observed after ADMSC transplantation throughout the study. Relative to the NP group without treatment, an increase in print area and max contact area, as well as intensity parameters were observed on day 28 ($p < 0.005$) and day 60 ($p < 0.02$) day of the experiment. When ADMSCs were transplanted 15 min after AM630 injection from day 21 of the experiment, we observed a decrease in print area to 90.2 % of the contralateral paw ($p < 0.002$ by day 0; Fig. 3c). Compared to the NP group without treatment, there was a reduction in print width to 93.8 % ($p < 0.001$), max intensity to 102.5 % ($p < 0.001$), and mean intensity to 100.3 % ($p < 0.005$; Fig. 3b, d, e) on day 21 of the study. Relative to the NP + ADMSC group, print area was lower on day 21 ($p < 0.05$), day 28 ($p < 0.05$) and day 60 ($p < 0.005$) of the experiment; max contact area was lower on days 28 and 60 of the experiment ($p < 0.05$). No other gait parameters were different when compared with the NP + ADMSC group. No statistically significant changes in static gait parameters were observed after transplantation of pre-AM630-ADMSCs against the values before NP modelling, as well as against the group of NP + ADMSCs.

Changes in the histologic structure of the sciatic nerve. The content of normal and damaged nerve fibres of the distal segment of the sciatic nerve of the experimental groups was assessed by the state of the myelin sheath and the location of the axial cylinder in the nerve fibre. Normal nerve fibres were clearly differentiated axial cylinders around which a uniformly stained myelin sheath with clear boundaries was observed. Damaged nerve fibres were characterized by swelling, vacuolated degeneration of myelin sheath, blurring of nerve fibre boundaries, with the axial cylinder displaced to the periphery or undetectable on histological sections (Fig. 4).

The contents of normal and damaged nerve fibres in the distal segment of the sciatic nerve in the NP group without treatment on day 21 of the experiment were 13 [11.75; 14] % and 87 [86; 87.5] %, respectively (Table 2; Fig. 4c).

After ADMSC transplantation, the proportion of preserved nerve fibres was statistically significantly higher in the distal segment of the sciatic nerve on day 21 of the study compared to the NP group without treatment ($p = 0.002$; Fig. 4a).

After ADMSC injection against the background of pharmacological blockade of CB_2-receptors in the soft tissues of the sciatic nerve transection area on day 21 no statistically significant changes in the content of normal and damaged nerve fibres were observed in comparison with the NP group without treatment ($p = 0.326$), but their number was significantly lower in comparison with the NP + ADMSC group ($p = 0.004$).

By day 90 of the study, 74 [72; 74] % damaged nerve fibres and 13 [11; 14] % undamaged nerve fibres were ob-

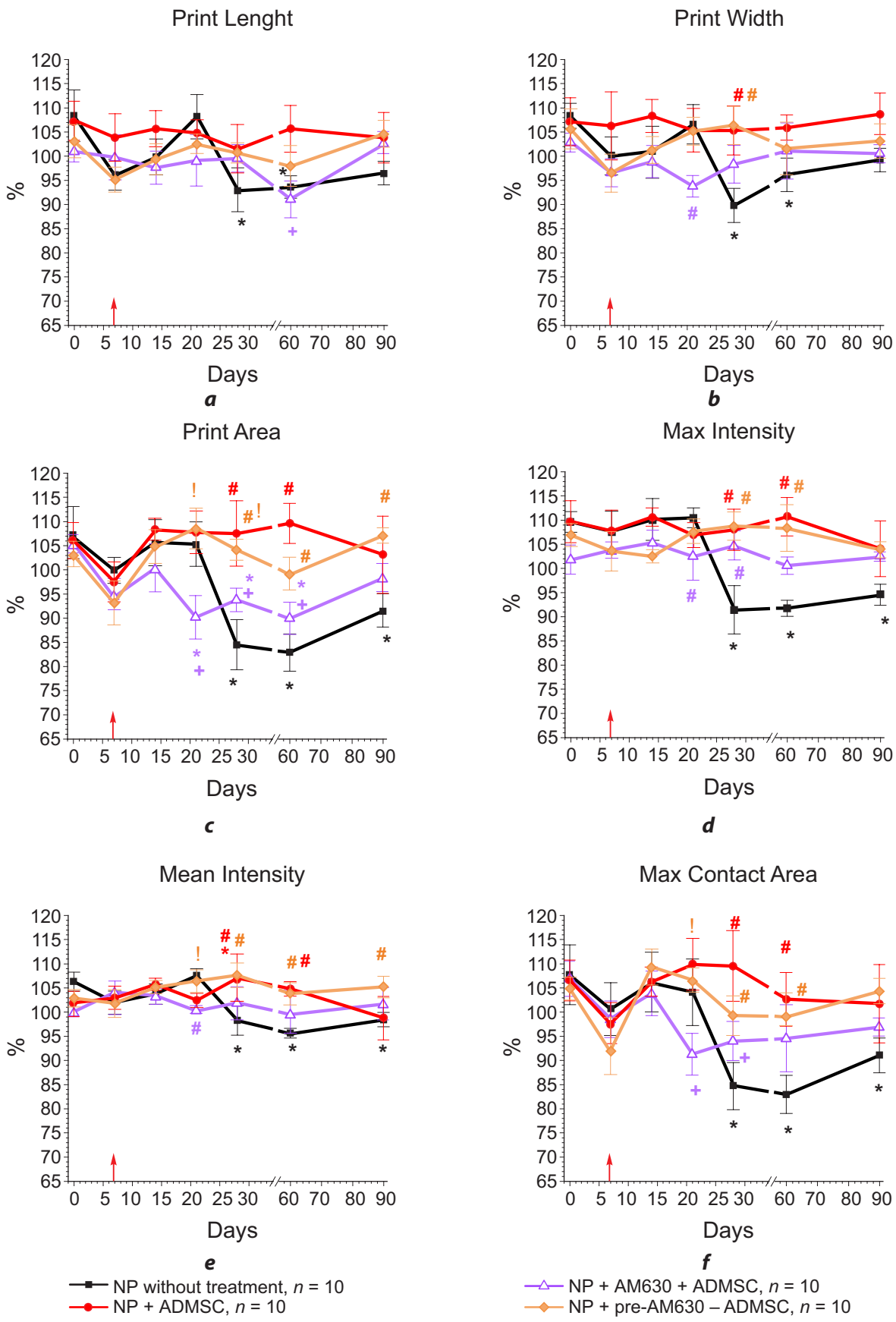
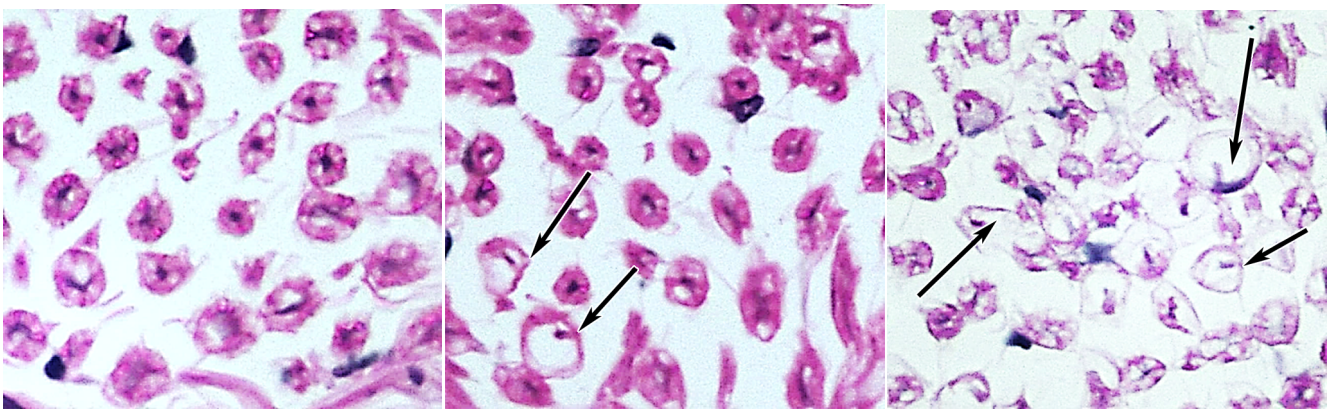


FIG. 3.

*Changes in static gait parameters: print length and print width (**a**, **b**), print area (**c**, **f**), as well as intensity parameters (**d**, **e**) – in rats after NP modelling, ADMSC transplantation on the background of pharmacological blockade of CB_2-receptors by AM630 antagonist. Arrow indicates transplantation time; * – p < 0.05 to values before NP modelling; # – p < 0.05 to NP group without treatment; + – p < 0.05 to NP + ADMSC; ! – p < 0.05 to the group of NP + AM630 + ADMSC*

served in NP without treatment (Table 2). After ADMSC transplantation, on day 90 of the study, the number of damaged nerve fibres was statistically significantly lower than that in NP without treatment ($p = 0.001$).

After ADMSC injection against the background of local administration of AM630 antagonist on day 90 a decrease in the proportion of damaged nerve fibres was observed in comparison with the NP group without treat-



a *b* *c*

FIG. 4.

Myelin nerve fibres of rat sciatic nerve: ***a*** *– preserved nerve fibres of the distal sciatic nerve fragment of an animal from the group after injection of ADMSCs, day 21;* ***b*** *– presence of dystrophically altered nerve fibres (arrows) in the distal sciatic nerve fragment of an animal from the group of NP + preAM630-ADMSCs, day 21;* ***c*** *– presence of dystrophically altered nerve fibres (arrows) in the distal sciatic nerve fragment of the group of NP animals without treatment. Hematoxylin and eosin staining, magnification ×400*

TABLE 2

STRUCTURE OF NORMAL AND DAMAGED NERVE FIBRES OF THE DISTAL SEGMENT OF THE SCIATIC NERVE OF THE EXPERIMENTAL GROUPS ON DAYS 21 AND 90 OF THE STUDY

Group	% of normal nerve fibers	% of damaged nerve fibers
	Day 21 of the study	
Untreated NPs	13 [11.75; 14]	87 [86; 87.5]
NP + ADMSCs	86 [84; 86]*	14 [13; 16]
NP + AM630 + ADMSCs	48 [48; 48]$^{\#}$	52 [51; 52]$^{\#}$
NP + pre-AM630-ADMSCs	55 [49; 55]	45 [38; 51]
	Day 90 of the study	
Untreated NPs	26 [25; 28]	74 [72; 74]
NP + ADMSCs	61 [61; 63]*	37 [37; 39]*
NP + AM630 + ADMSCs	37 [26; 37]$^{\#}$	63 [60; 74]$^{\#}$
NP + pre-AM630-ADMSCs	65 [56.5; 65]*;$^{!}$	35 [32; 43.5]*;$^{!}$

Note. * – $p < 0.05$ to NP without treatment; $^{\#}$ – $p < 0.05$ to NP + MSC; $^{!}$ – $p < 0.05$ between preincubation and local injection (Kruskal – Wallis criterion).

ment (p = 0.008) and an increase in comparison with the NP + ADMSC group (p = 0.036). Pre-AM630-MSC transplantation resulted in a decrease in the number of damaged nerve fibres and an increase in the number of normal nerve fibres by day 90 of the study compared to NP without treatment (p = 0.042) and was comparable to the NP + ADMSC group.

DISCUSSION

ADMSC transplantation is currently positioned as an effective method to reduce the progression of pain syndrome of inflammatory [21] and neurogenic origin [6–11]. ADMSC effectiveness in suppressing nocicetptive sensitivity disorders, such as mechanical and thermal hyperlagia and allodynia has been demonstrated in experimental models of chronic ligation of the sciatic nerve [8] and infraorbital nerve [9] in rodents, as well as in models of partial [8, 10] and complete traumatic nerve injury [6, 7], a model of streptozotocin-induced diabetic polyneuropathy [11]. However, the mechanisms of ADMSC realised effects are still being studied. The endocannabinoid system is known to be involved in the inhibition of nociceptive signal transduction and transmission in both peripheral tissues and the central nervous system, and modulation of its components serves as one of the targets of pain relief in neuropathic pain. Conversely, ADMSCs express components of the endocannabinoid system, in particular, CB_1 and CB_2 receptors [14–16], the activation of which increases their secretion of such factors as vascular endothelial growth factor, transforming growth factor-beta and hepatocyte growth factor [16]. Moreover, activation of the CB_2 receptor on MSC leads to decreased production of interleukin (IL) 6, IL-8 and tumour necrosis factor α, as well as increased IL-10 [13, 15, 22]. From the evidence available in the literature, the authors suggested that CB_2 receptors mediate immunomodulatory and antinociceptive effects of ADMSCs.

The data obtained in this study indicate that pharmacological blockade of CB_2-receptors both on the membranes of MSCs and in the soft tissues surrounding the site of sciatic nerve injury, reduces the antinociceptive effect of ADMSCs at their transplantation into the site of sciatic nerve transection in rats, which was revealed by the lack of MWT and TWL recovery in the respective groups of animals, as well as worsening of mechanical and thermal hyperalgesia at the later stages of the study (Fig. 1a, b). Blockade of CB_2-receptors on MSCs significantly slowed down SFI recovery (Fig. 2d), and their deactivation in tissues in the area of sciatic nerve transection completely abolished the SFI restoring effect of MSCs (Fig. 2d). The abovementioned facts in aggregate allow to assume at least weakening of antinociceptive properties of ADMSC in response to blockade of the indicated receptor.

The histological study of the sciatic nerve distal segment revealed the protective effect of MSC, which was manifested in weakening of degenerative changes of nerve fibres. Both methods of CB_2 receptor blockade abolished the mentioned protective effect of ADMSC against the injured nerve fibres. The content of the latter on day 21 of the experiment did not differ in these groups from that in untreated animals (Table 2). In addition, at late study periods (90 days), CB_2-receptor blockade in ipsilateral soft tissues was accompanied by a higher proportion of injured nerve fibres than in the NP + pre-AM630-MSC group (Table 2). In summary, the results of this study indicate the involvement of CB_2 receptors in the mechanisms of the ADMSCs protective effects. In this case CB_2-receptor blockade applied to peripheral nerve fibres has a greater effect on the MSC reparative potential, and pharmacological inactivation of these receptors on stem cells themselves – to their antinociceptive action.

CONCLUSION

It has been experimentally confirmed that pharmacological blockade of CB_2-receptors both on ADMSC membranes and in the peripheral nerve injury area decreases antinociceptive and reparative effect of ADMSC at their local transplantation. This indicates the direct participation of these receptors in the protective effects realised by MSCs. Further study of the CB_2-receptor stimulation effect will allow to estimate the degree to which the antinociceptive and reparative effects of ADMSCs are enhanced when they have been transplanted into the site of peripheral nerve injury.

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Conflict of interest

The authors declare no conflict of interest.

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Information about the authors

Anna-Maria V. Yerofeyeva – Postgraduate, Junior Research Officer at the Laboratory of Biological Modeling, Institute of Physiology, National Academy of Sciences of Belarus, e-mail: amyerofeyeva@zoho.eu, https://orcid.org/0000-0002-5499-5950

Sergei V. Pinchuk – Cand. Sc. (Biol.), Leading Research Officer at the Laboratory of Molecular Cell Biology, Institute of Biophysics and Cell Engineering, National Academy of Sciences of Belarus, e-mail: pinchuksv@mail.ru, https://orcid.org/0000-0002-5499-5950

Svetlana N. Rjabceva – Cand. Sc. (Med.), Head of the Laboratory «Center of Electron and Light Microscopy», Institute of Physiology, National Academy of Sciences of Belarus, e-mail: ryabceva@physiology.by, https://orcid.org/0000-0001-5960-3656

Alla Yu. Molchanova – Cand. Sc. (Biol.), Leading Research Officer at the Laboratory of Biological Modeling, Institute of Physiology, National Academy of Sciences of Belarus, e-mail: kjordknits@gmail.com, https://orcid.org/0000-0001-5053-6602

Authors' contribution

Anna-Maria V. Yerofeyeva – conducting a study, analysing data, writing a text.
Sergei V. Pinchuk – conducting a study.
Svetlana N. Rjabceva – supervision of the R&D work, text editing.
Alla Yu. Molchanova – study concept, approval of the final version of the study manuscript.

NEUROLOGY AND NEUROSURGERY

ACCUMULATION OF AGGREGATED ALPHA-SYNUCLEIN IN NEURAL TISSUE STRUCTURES IN NEURODEGENERATIVE DISEASES

Salkov V.N.
Voronkov D.N.

Research Center of Neurology
(Volokolamskoye highway 80,
Moscow 125367, Russian Federation)

Corresponding author:
Vladimir N. Salkov,
e-mail: vla-salkov@yandex.ru

ABSTRACT

A critical analysis of the literature on the structure and properties of alpha-synuclein under physiological and pathological conditions is presented, when the conformation of this protein changes, which contributes to its aggregation and changes in localization features in brain structures in such neurodegenerative diseases as Parkinson's disease, dementia with Lewy bodies, multiple systemic atrophy and Alzheimer's disease. It has been shown that the toxic effect of conformationally altered alpha-synuclein can indirectly affect the functions of neurons due to its interaction with neuroglial cells, primarily microglia and astrocytes, and can also modulate the aggregation and expression of other proteins that are functionally important for the development of neurodegeneration.
Further study of the mechanisms of interaction of conformationally altered alpha-synuclein with other proteins and clarification of the relationship between its accumulation in brain structures and neuronal dysfunction remains relevant for modern neurology.
Literature search was carried out in the "PubMed" and "eLIBRARY" databases.

Key words: *conformations of alpha-synuclein, neurons, neuroglia, Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, Alzheimer's disease*

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НАКОПЛЕНИЕ АГРЕГИРОВАННОГО АЛЬФА-СИНУКЛЕИНА В СТРУКТУРАХ НЕРВНОЙ ТКАНИ ПРИ НЕЙРОДЕГЕНЕРАТИВНЫХ ЗАБОЛЕВАНИЯХ

Сальков В.Н.,
Воронков Д.Н.

ФГБНУ «Научный центр неврологии»
(125367, г. Москва,
Волоколамское шоссе, 80, Россия)

Автор, ответственный за переписку:
Сальков Владимир Николаевич,
e-mail: vla-salkov@yandex.ru

РЕЗЮМЕ

Представлен критический анализ литературы о строении и свойствах альфа-синуклеина в физиологических условиях и в условиях патологии, когда изменяется конформация этого белка, что способствует его агрегации и изменению особенностей локализации в структурах головного мозга при таких нейродегенеративных заболеваниях, как болезнь Паркинсона, деменция с тельцами Леви, множественная системная атрофия и болезнь Альцгеймера.
Показано, что токсическое действие конформационно изменённого альфа-синуклеина может опосредованно влиять на функции нейронов вследствие его взаимодействия с клетками нейроглии, в первую очередь с микроглией и астроцитами, а также может модулировать агрегацию и экспрессию других белков, функционально значимых для развития нейродегенерации.
Дальнейшее исследование механизмов взаимодействия конформационно измененного альфа-синуклеина с другими белками и уточнение взаимосвязи между его накоплением в структурах головного мозга и дисфункцией нейронов остаются актуальными для современной неврологии.
Поиск литературы проводился в базах данных «PubMed» и «eLIBRARY».

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INTRODUCTION

Most neurodegenerative diseases associated with old age are characterised by morphochemical signs of proteinopathies, i.e. impairment accompanied by disturbances in the structure of certain proteins (alpha-synuclein, tau-protein, beta-amyloid, etc.) and their metabolism [1]. The most common diseases in this group are Parkinson's (PD) and Alzheimer's (AD) diseases [2]. At their core are proteinopathies associated with impaired aggregation of alpha-synuclein, a protein that accumulates in brain structures [3]. In experiments on laboratory animals and studies using brain autopsy material in the mentioned nosological forms of alpha-synucleinopathies, the peculiarities of changes in the structure of this protein have been revealed [4], but the peculiarities of its accumulation in nervous tissue in these and other forms of alpha-synucleinopathies have been studied insufficiently.

THE AIM OF THE STUDY

To characterise the peculiarities of aggregated alpha-synuclein accumulation in neural tissue structures in neurodegenerative diseases based on the analysis of literature sources.

GENERAL CHARACTERIZATION OF ALPHA-SYNUCLEIN AND ITS BIOCHEMICAL PROPERTIES

Alpha-synuclein is a small protein (molecular mass not exceeding 40 kilodaltons) that is synthesised mainly in the cytoplasm and presynaptic terminals of neurons of the human and vertebrate central nervous system [5]. The structure of this protein distinguishes between a hydrophobic central domain, known as the non-amyloid component, and two terminal areas: one is the N-terminal, which exhibits amphipathic properties and interacts with cell membranes, and the other area is the negatively charged C-terminal, which contains several phosphorylation sites [6]. The non-amyloid component accumulates in high concentrations in senile plaques during AD [7], the N-terminus has the ability to undergo most of the known mutations associated with PD – A53T, A30P as well as E46K, G51D and H50Q [8], and phosphorylation (by serine) of site-129 of the C-terminus causes impaired polymerisation and aggregation of alpha-synuclein with subsequent formation of intracellular inclusions in brain structures, which is characteristic of both PD and other alpha-synucleinopathies (dementia with Lewy bodies (DLB), multiple system atrophy (MSA), etc.) [9]. Compared to other synucleins with a similar structure to alpha-synuclein, it has a significant level of expression in brain structures (compared to gamma-synuclein) and is capable of aggregation (compared to beta-synuclein, which has no non-amyloid component in its structure) [10].

Alpha-synuclein is characterized by structural diversity. Thus, while in physiological conditions it is located in cells in the state of a monomer (non-toxic low-molecular polypeptide capable of polymerisation reactions), in the above-mentioned neurodegenerative diseases its structure changes, which promotes its self-organisation first into oligomers (dimers, trimers, tetramers), which consist of single monomers, and later, by transforming all or part of the previously unstructured polypeptide into well-defined β-sheet-rich secondary structures (insoluble filaments or fibrils) [11], penetrating into other neurons, "recruiting" endogenous alpha-synuclein into them, and forming new insoluble aggregates [12]. Due to the ability of pathological forms of alpha-synuclein to be transmitted from neuron to neuron, alpha-synucleinopathies are often correlated with prion diseases [13] and it is assumed that, by analogy with prion diseases, which are caused by different strains of prions that differ in their biochemical characteristics (ability to be cleaved by proteinase K, glycosylation capacity, etc.), different alpha-synucleinopathies may also be associated with different "strains" of pathological alpha-synuclein [14]. As evidence, the observation that the pathological process in the limbic cortex, as well as in other areas of the brain, develops much faster in DLB than in PD [15] is provided, and it is suggested that this may be due to the fact that these diseases are caused by different "strains" of alpha-synuclein [6]. Such evidence is not conclusive, however, since to date no biochemical differences have been demonstrated between the types of alpha-synuclein that accumulates in cortical structures during PD and DLB. It would appear that a more significant argument in favour of the "prion hypothesis" is the fact that wild-type alpha-synuclein contains glutamate at amino acid residue 46 and lysine in cases of hereditary forms of PD [16]. The change in this residue is sufficient to prevent alpha-synuclein conformation, which accumulates in hereditary forms of PD, from adopting any of the conformations that alpha-synuclein protofibrils have in MSA.

Alpha-synuclein under physiological conditions can be found in the cell in a membrane-bound and soluble stable state; the latter was discovered by examining the proteins in human erythrocytes using analytical centrifugation [17, 18]. In cells, alpha-synuclein binds to lipid membrane structures such as liposomes, lipid droplets and lipid rafts, which require the presence of oxidised lipids [19] such as phosphatidylserine or phosphatidylinositol and involves membrane interaction with lysines found in the structure of this protein. Binding to negatively charged lipid membrane structures causes alpha-synuclein to acquire a helical conformation [20]. This form of alpha-synuclein is thought to be observed in cells as two variants: a single elongated α-helix and a broken α-helix represented by two antiparallel non-interacting helices [21]. Molecular modelling methods have been used to reveal that the formation of an elongated α-helix is promoted by the interaction of alpha-synuclein with membrane structures of large diameter (~100 nanometres or more), while its interaction with membrane structures of smaller diameter promotes the formation of a broken α-helix

[22]. The above spiral shapes appear to be characteristic of native alpha-synuclein under physiological conditions, whereas under pathological conditions it takes the form of a β-sheet. This alpha-synuclein conformation is associated with the processes of aggregation of this protein, formation of fibrils and their deposition in Lewy's bodies, which are formed in the black substance neurons of the brain as a result of PD [23]. The β-sheet alpha-synuclein conformation is thought to be neurotoxic, but the exact genesis of this form remains unclear [24].

ALPHA-SYNUCLEIN TOXICITY AND ITS INTERACTION WITH NEURONAL DYSFUNCTION

Serine phosphorylated alpha-synuclein (α-Syn-p129) was found experimentally to be highly toxic regardless of the size of its aggregates accumulating in nerve cells and neuropil [25]. Accordingly, it was revealed that if both single oligomers of α-Syn-p129 and its larger aggregates (fibrils) obtained from PD patients were stereotactically injected into the brain shell structures of adult baboons, which in turn were isolated from Lewy's bodies of autopsy brains of individuals with PD (brain donation programme of the Brain Bank 'GIE NeuroCEB'), then in 2 years in experimental animals, regardless of the size of alpha-synuclein fractions administered to them, neurodegeneration developed in the compact part of the black substance of the brain, leading to the death of not only dopamine neurons, but also other neurons. The cytotoxicity of alpha-synuclein may be attributed to its ability to bind to large curvature membranes [26], which is a common property for amphipathic α-helices and is explained by the fact that, compared to flattened membranes, large curvature membranes provide a higher density of binding sites for helix-shaped proteins when interacting with lipid membrane structures [27]. Furthermore, it is suggested that in impairment, alpha-synuclein, by acting on mitochondrial membranes, may cause their fragmentation [28]. At the same time, binding mainly to the inner mitochondrial membrane, it can interact with complex I, which reduces mitochondrial activity and increases autophagy (mitophagy) of these structures [29].

The neurotoxicity of α-Syn-p129 is also manifested in the fact that it can induce the influx of calcium ions into the cell both directly, through the formation of pore-like ring structures in the plasma membrane [30], and indirectly, through the activation of potential-dependent N-type calcium channels [31], as a result of which the concentration of calcium ions in neurons increases, and the cell membrane of the nerve cell depolarises, which leads to the release of neurotransmitters.

The toxicity of alpha-synuclein conformation may be associated with the loss of the helical conformation by this protein [32]. The relative stability of helicality was demonstrated in an in vitro experiment in which the addition of small single-layer negatively charged lipid vesicles did not induce significant conformational changes in the native alpha-synuclein tetramer [17]. However, exposure to a number of factors, such as genetic mutations, aging, inflammatory process, and environmental toxins, can contribute to the fact that it loses the orderliness of its structure and consequently loses its helical conformation, taking the form of an insufficiently structured protein, i.e. β-sheet [33]. Such alpha-synuclein conformation is adopted in neurodegenerative diseases, the development of which is induced by oxidative stress and accumulation of nitric oxide (II) in nervous tissue [34]. Alpha-synuclein oligomers formed during oxidative stress are phosphorylated and as a result hydrogen peroxide molecules are released [35]. This process results in the presence of transition metal ions exhibiting redox properties: Fe (II), Cu (I) and others. Metal ions, binding to the alpha-synuclein molecule, form specific oxygen bridges that are destabilised when the alpha-synuclein conformation changes and oligomerises. This releases superoxide anion, which subsequently undergoes reversible conversion to hydrogen peroxide [36]. Nitrosative stress, in which the formation of active nitrogen forms exceeds the possibilities of their neutralisation or elimination, leads to the formation of covalent bonds between nitric oxide (II) and specific thiol groups of proteins and can be considered as a probable mechanism, contributing to NO-induced incorrect aggregation of various proteins, including alpha-synuclein [37], which is confirmed by the detection of nitrosylated alpha-synuclein in Lewy's bodies, which are localised in brain structures during PD [38].

The relationship between the accumulation of highly toxic α-Syn-p129 in neurons and their dysfunction, however, remains unclear [39]. The number of neurons in the black substance significantly decreases even before the development of the main clinical symptoms of PD or at early stages of the disease (from 50 to 90 %) [40], which, according to studies of the relationship between neuronal loss and the accumulation of α-Syn-p129 in dopamine neurons of the black substance in this impairment, suggests that its accumulation in brain structures is not as significant as the loss of nerve cells [39]. In other nervous system entities, such as the enteric nervous system, the clinical manifestations of Parkinsonism are not related to neuronal death but to the accumulation of α-Syn-p129 in nervous tissue [41]. Simultaneously, transgenic mice with high expression of wild-type alpha-synuclein showed impaired behavioural responses associated with changes in olfaction, intestinal peristalsis and motor activity, but they did not reveal morphological signs of neurodegenerative process [42]. Besides, morphological signs of alpha-synuclein accumulation were observed in autopsy material of the midbrain of elderly people who died from intercurrent diseases, but no neurological symptoms were revealed in these people during their lifetime [43]. Accordingly, it is suggested that the accumulation of aggregated alpha-synuclein in the brains of neurologically healthy individuals is an adaptive response of the organism, and the increase in morphochemical indicators of the neurodegenerative process

in the brains of PD patients is the result of the accumulation of toxic α-Syn-p129.

Consequently, despite the high toxicity of α-Syn-p129, the excessive accumulation of this protein in nervous tissue alone is clearly insufficient for the development of neurodegeneration, and its actual role in the development of this process remains to be determined.

The information about the structural characteristics of aggregated alpha-synuclein and its neurotoxicity outlined above was obtained mainly in experimental animal studies. Accordingly, they do not provide a complete picture of the morphological basis and possible mechanisms of pathogenesis of alpha-synucleinopathies. Meanwhile, these data significantly expand and supplement the data of pathomorphological studies performed by immunohistochemical methods on autopsy material of patients with alpha-synucleinopathies.

ALPHA-SYNUCLEIN LOCALIZATION AND ACCUMULATION IN PARKINSON'S DISEASE

Localisation of α-Syn-p129 during PD, considered as an alpha-synucleinopathy, is observed not only in the central (CNS) but also in the peripheral nervous system [44], and in the latter this protein often starts to accumulate earlier than in the CNS [45]. This can probably explain the earlier appearance during PD of symptoms indicating peripheral cranial nerve damage (hyposmia, reduced visual contrast and colour discrimination), symptoms of gastrointestinal and cardiovascular dysfunction, and the later appearance of symptoms of motor disorders, which are considered to be the main clinical manifestations of the disease [44].

The sequence of α-Syn-p129 deposition in different parts of the nervous system revealed in pathomorphological studies formed the basis for the scheme of clinical and morphological stages of PD, which considered the involvement of both peripheral and central parts of the nervous system in the pathological process and postulated the spread of pathological changes from caudal brain formations to cortical formations [46]. Furthermore, the "double hit" hypothesis has been proposed to explain the progression of PD in terms of matching the sequence of stages of the clinical picture of the disease with the morphological changes observed [47]. Based on this hypothesis, an unknown neurotropic pathogen (presumably a virus) can penetrate through the olfactory tract and the fibres of the vagus nerve innervating the digestive system into brain structures: in the first case into the temporal lobe, in the second case into the medulla oblongata, pons cerebelli and mesencephalon. In the latter formation, namely in the compact part of the black substance, there is a secondary accumulation of α-Syn-p129 in the form of Lewy's bodies localised both in the cytoplasm of neurons and outside the cells, resulting in the death of dopamine neurons. Initially, α-Syn-p129 accumulates either in the olfactory bulbs or in the dorsal motor nucleus of the glossopharyngeal and vagus nerves. The hypothesis was confirmed by cross-sectional analysis of pathological changes in brain samples from individuals with PD who died as a result of intercurrent diseases. The scheme of PD stages and the "double hit" hypothesis, despite its popularity, have been repeatedly and justifiably criticised by the scientific community. Consequently, α-Syn-p129 accumulations were revealed simultaneously in various brain formations, thus refuting the possibility of this protein spreading only in the direction from the medulla oblongata to the mesencephalon and cerebrum structures [48]. Besides, the concept did not explain the presence of oligomeric alpha-synuclein in elevated amounts in the plasma and liquor of PD patients [49]. Finally, the scheme to define the clinical and morphological stages of PD was questioned by the author himself [50].

It is currently believed that impaired alpha-synuclein aggregation, and subsequently its accumulation, occurs simultaneously in several structures of the central and peripheral nervous system already at the latent stage of the neurodegenerative process [51]. Furthermore, single-photon emission computed tomography revealed that different clinical scenarios of PD course correspond to the involvement of different structures of the nervous system in the pathological process [52], and the accumulation of α-Syn-p129 in the nervous tissue is not always the cause of dopamine neuron death in this disease [53]. The accumulation of α-Syn-p129, however, can activate microglia whose activity level corresponds to the degree of neurotoxicity [54], and, in addition, oligomeric alpha-synuclein enhances the phagocytic function of microglia [55].

Consequently, α-Syn-p129, which accumulates in brain structures during PD, can apparently have the same toxic effect on dopamine neurons of the black substance as other pathogenic factors: metal ions, pesticides, etc. [56].

ALPHA-SYNUCLEIN ACCUMULATION IN OTHER NEURODEGENERATIVE DISEASES

Clinically, DLB is very similar to dementia during PD [57], and they are distinguished using the "1-year rule": if dementia occurs against the background of PD at least 1 year after diagnosis, the case is considered as 'dementia in Parkinson's disease'; if dementia precedes or occurs simultaneously with the appearance of clinical symptoms of Parkinsonism or it develops within a year after their appearance, DLB is diagnosed. Both diseases are characterised by the accumulation of aggregated alpha-synuclein in the form of Lewy bodies and Lewy neurites, which in autopsy brains of individuals with PD are revealed only in brainstem and limbic system structures, while in DLB and dementia developed on the background of PD, they are also found in the neocortex [58]. Positron emission tomography and pathomorphology revealed that cortical atrophy in DLB was more pronounced

than in dementia developed on the background of PD [59]. However, the number of Lewy bodies in the limbic region, especially in the CA2 field of the hippocampus, and in the temporal region of the neocortex was significantly higher during DLB than in dementia developed on the background of PD [60], and in the latter case there was significantly higher death of dopamine neurons in the black substance [61]. Moreover, DLB is characterised by loss of dopamine neurons in the medioventral segment of the black substance, whereas dementia in PD is characterised by loss of dopamine neurons in its dorsolateral segment.

Along with the fact that aggregated alpha-synuclein in the structures of nervous tissue was revealed in PD and DLB, it was also observed in MSA [62], but, unlike PD and DLB, alpha-synuclein deposits in MSA were mainly accumulated in the cytoplasm and nuclei of oligodendrocytes, and they were also observed in the bodies and outgrowths of neurons [63]. Autopsy samples from the cerebrum ($n = 14$) and spinal medulla ($n = 11$) of MSA patients revealed alpha-synuclein inclusions in the cytoplasm of glial cells in structures of the motor cortex, putamen, pontine, medulla oblongata, and suprasegmental centres of the autonomic nervous system [64], as well as in the caudate nucleus, external pallidum, black substance, locus coeruleus, and cerebellum [65]. Considering that alpha-synuclein is not expressed in significant amounts in oligodendrocytes under physiological conditions, it is not clear how its aggregated form accumulates in the cytoplasm of glial cells in impairment [66]? In addressing this issue, some authors believe that pathological inclusions in neuroglia can be formed on the basis of aggregated alpha-synuclein, which is released from neurons and then captured by neighbouring astrocytes [67], while others suggest that the cause of accumulation of alpha-synuclein aggregates in oligodendrocytes is *SNCA* gene activation [66]. In addition, in vivo experiments demonstrated that oligodendrocytes can internalize aggregated alpha-synuclein when administered to mice [68].

AD in a significant number of cases (up to 50 %) manifests accumulation of aggregated alpha-synuclein in brain structures [69], mainly in the amygdala [70], albeit its accumulation is not considered a characteristic pathomorphological sign of this disease. For instance, on autopsy brains by immunohistochemical methods, Lewy bodies were found during AD in 10 out of 22 cases [71] and were detected in the bodies rather than in the outgrowths of neurons [72]. It is suggested that alpha-synuclein during AD directly interacts with Aβ-peptide and tau-protein, and this contributes to the mutual aggregation of these proteins [73]. Meanwhile, it has been also revealed that injection of tau-protein grains and preformed fibrils derived from purified recombinant alpha-synuclein into the hippocampus and cortical plate region of laboratory mice significantly increases the number of tau-positive neurons but does not affect the number of alpha-synuclein-positive neurons [74].

Consequently, alpha-synuclein can modulate the aggregation and expression of other proteins functionally relevant for the development of neurodegeneration during AD, but the effect of these proteins on alpha-synuclein aggregation has not been proven to date.

CONCLUSION

It has therefore been established to date that alpha-synuclein under physiological conditions is found in the cytoplasm of neurons and presynaptic terminals of axons. In impairment it can change its conformation and acquire neurotoxic properties, which are being realised as a result of its interaction with elements of neuroglia, primarily with microglial cells and astrocytes. Moreover, it can modulate the expression of other neuronal proteins functionally relevant in neurodegenerative diseases such as PD, DLB, MSA and AD. Along with this, the data about the accumulation of aggregated alpha-synuclein in the structures of nervous tissue do not allow us to fully establish its role in the pathogenesis of these diseases, but this seems possible if we continue to study the mechanisms of its interaction with other proteins and clarify the relationship between its accumulation in brain structures and neuronal dysfunction.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Vladimir N. Salkov – Dr. Sc. (Med.), Senior Research Officer at the Laboratory of Neuromorphology of Brain Institute, Research Center of Neurology, e-mail: vla-salkov@yandex.ru, https://orcid.org/0000-0002-1580-0380

Dmitry N. Voronkov – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Neuromorphology of Brain Institute, Research Center of Neurology, e-mail: voronkov@neurology.ru, https://orcid.org/0000-0001-5222-5322

ONCOLOGY

CYTOTOXIC EFFECT OF THE VV-GMCSF-LACT ONCOLYTIC VIRUS AGAINST 3D CULTURES OF HUMAN GLIOBLASTOMA CELLS U-87 MG

Dymova M.A. [1],
Shnaider T.A. [2],
Chechetkina S.A. [3],
Petrov G.O. [1],
Malysheva D.O. [1],
Drokov D.V. [3],
Ageenko A.B. [1],
Vasileva N.S. [1],
Richter V.A. [1],
Kuligina E.V. [1]

[1] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences (Akademika Lavrentieva Ave. 8, Novosibirsk 630090, Russian Federation)
[2] Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (Akademika Lavrentieva Ave. 10, Novosibirsk 630090, Russian Federation)
[3] Novosibirsk State University (Pirogova str. 2, Novosibirsk 630090, Russian Federation)

Corresponding author:
Maya A. Dymova,
e-mail: maya.a.rot@gmail.com

ABSTRACT

Background. *One of the promising methods of treating tumors is virotherapy, which is based on direct lysis of cancer cells by a virus and a virus-mediated antitumor immune response of the body. For the recombinant vaccinia virus strain VV-GMCSF-Lact, producing human GMCSF and the oncotoxic protein lactaptin, cytotoxic and antitumor effects were shown in experiments in vitro and in vivo, respectively, when using adherent cultures of U-87 MG human glioblastoma cells. 3D cultures are a more relevant tumor model than adherent models, as they more fully reflect the realistic scenario of cancer development, as well as the response of the tumor to anticancer therapy.*

The aim of the study. *To evaluate the cytotoxic effect of the oncolytic virus VV-GMCSF-Lact against 3D cultures of human glioblastoma U-87 MG.*

Materials and methods. *The following methods were used in the work: cultivation of 3D cell cultures, cytofluorometry, microscopic analysis, virus titration, and statistical analysis.*

Results. *U-87 MG cells were transduced with a lentiviral vector carrying the GFP reporter gene. The cytotoxicity of the VV-GMCSF-Lact virus (IC50) against the studied cells was 0.024 PFU/cell. U-87 MG cells were cultured under conditions for the formation of 3D structures. Microscopic analysis showed the oncolytic effect of the virus on the cells of 3D cultures as early as 24 hours after the start of incubation. Flow cytometry showed an increase in the granularity of glioblastoma cells under the action of the virus, which indicates active replication of the virus in the cells. The virus titer was 0.44 PFU/cell.*

Conclusion. *The recombinant VV-GMCSF-Lact virus has a cytotoxic effect on 3D human glioblastoma U-87 MG cell cultures and actively replicates in them. In the future, to test the oncolytic effect of VV-GMCSF-Lact, it is planned to use not only 3D human glioblastoma cultures, but also cerebral organelles obtained in the process of cocultivation of glioblastoma cells and induced human pluripotent cells.*

Key words: *glioblastoma, neurospheres, oncolytic virus, VV-GMCSF-Lact*

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ЦИТОТОКСИЧЕСКОЕ ДЕЙСТВИЕ ОНКОЛИТИЧЕСКОГО ВИРУСА VV-GMCSF-LACT В ОТНОШЕНИИ 3D-КУЛЬТУР КЛЕТОК ГЛИОБЛАСТОМЫ ЧЕЛОВЕКА U-87 MG

Дымова М.А. [1],
Шнайдер Т.А. [2],
Чечеткина С.А. [3],
Петров Г.О. [1],
Малышева Д.О. [1],
Дроков Д.В. [3],
Агеенко А.Б. [1],
Васильева Н.С. [1],
Рихтер В.А. [1],
Кулигина Е.В. [1]

[1] ФГБУН Институт химической биологии и фундаментальной медицины СО РАН (630090, г. Новосибирск, просп. Академика Лаврентьева, 8, Россия)
[2] ФГБНУ «Федеральный исследовательский центр Институт цитологии и генетики СО РАН» (630090, г. Новосибирск, просп. Академика Лаврентьева, 10, Россия)
[3] ФГАОУ ВО «Новосибирский государственный университет» (630090, г. Новосибирск, ул. Пирогова, 2, Россия)

Автор, ответственный за переписку:
Дымова Майя Александровна,
e-mail: maya.a.rot@gmail.com

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РЕЗЮМЕ

Обоснование. *Одним из перспективных методов лечения опухолей является виротерапия, в основе которой лежит прямой лизис вирусом опухолевых клеток и вирус-опосредованный противоопухолевый иммунный ответ организма. Для рекомбинантного штамма вируса осповакцины VV-GMCSF-Lact, продуцирующего GMCSF человека и онкотоксический белок лактаптин, показано цитотоксическое и противоопухолевое действие в экспериментах in vitro и in vivo соответственно при использовании адгезивных культур клеток U-87 MG глиобластомы человека. 3D-культуры являются более релевантной моделью опухоли в сравнении с адгезивными моделями, так как более полно отражают реалистичный сценарий развития опухолевого процесса, а также ответа опухоли на противоопухолевую терапию.*

Цель исследования. *Оценка цитотоксического действия онколитического вируса VV-GMCSF-Lact в отношении клеток 3D-культур глиобластомы человека U-87 MG.*

Методы. *В работе использовались следующие методы: культивирование 3D-культур клеток; цитофлуориметрия; микроскопический анализ; титрование вируса; статистическая обработка данных.*

Результаты. *Клетки U-87 MG были трансдуцированы лентивирусным вектором, несущим ген GFP. Цитотоксичность вируса VV-GMCSF-Lact (IC50) в отношении исследуемых клеток составила 0,024 БОЕ/клетку. Далее клетки U-87 MG культивировали в условиях формирования 3D-структур. С помощью микроскопического анализа показано онколитическое действие вируса на клетки 3D-культур уже спустя 24 часа после начала инкубации. Методом проточной цитофлуориметрии показано увеличение гранулярности клеток глиобластомы под действием вируса, что указывает на активную репликацию вируса в клетках. Титр вируса составил 0,44 БОЕ/клетку.*

Заключение. *Рекомбинантный вирус VV-GMCSF-Lact оказывает цитотоксическое действие на 3D-культуры клеток глиобластомы человека U-87 MG, активно реплицируется в них. В дальнейшем для тестирования онколитического действия VV-GMCSF-Lact планируется использовать не только 3D-культуры глиобластомы человека, но и церебральные органоиды, полученные в процессе сокультивирования клеток глиобластомы и индуцированных плюрипотентных клеток человека.*

Ключевые слова: *глиобластома, нейросферы, онколитический вирус, VV-GMCSF-Lact*

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INTRODUCTION

Human glioblastoma (GB) or astrocytoma of grade IV malignancy is one of the most common types of malignant primary brain tumours, being characterised by high invasiveness, heterogeneity and aggressiveness of the tumour process. With a conventional treatment regimen (the standard therapy for GB is the Stupp protocol) including surgical resection, radio- and chemotherapy with temozolomide, the survival rate of patients diagnosed with glioblastoma is only 26.5 %, with an overall survival rate of more than 10 years of 0.71 % [1]. Actually, the mean life expectancy after diagnosis is about 15 months, with a survival rate of usually no more than 3 months in the absence of therapy. The disease is more common in men than in women, and the average age of patients at the time of diagnosis is 64 years. As a result, the development of new therapeutic approaches for such difficult-to-treat cancers is urgently needed. One of the promising methods of tumour treatment is virotherapy, which is based on direct lysis of tumour cells by virus and virus-mediated anti-tumour host immune response. The anti-tumour efficacy of recombinant viruses can be enhanced by inserting genes of oncotoxic and immunomodulatory proteins into their genome.

The recombinant VV-GMCSF-Lact strain of smallpox vaccine virus producing human granulocyte-macrophage colony-stimulating factor (GMCSF) and the oncotoxic protein lactaptin was used in this study [2]. VV-GMCSF-Lact has previously been shown to have an anti-tumour effect against breast cancer and is currently in clinical trials (ClinicalTrials.gov: NCT05376527). Nevertheless, the activity spectrum of this virus is much broader: in particular, its cytotoxic and anti-tumour effects were revealed in *in vitro* and *in vivo* trials, respectively, using adherent cultures of human glioblastoma U-87 MG [3]. 3D cultures are known to be a more relevant tumour model as they allow a more complete reproduction of the tumour development scenario as well as the response to anti-tumour therapy.

THE AIM

Assessment of the oncolytic action of VV-GMCSF-Lact against 3D-cultured human glioblastoma U-87 MG cells. In order to better visualise the cytotoxic effect of the recombinant virus, in this study the human glioblastoma U-87 MG cells pre-transduced with lentivirus carrying a *GFP* reporter gene were used.

METHODS

Cell cultivation

U-87 cells transduced with lentivirus carrying *GFP* gene were cultured in complete growth medium DMEM/F12 supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1X MEM essential amino acid solution and antibiotic solution (100 U/ml penicillin, 100 mg/ml streptomycin sulfate) at 37 °C in a CO_2 incubator. To obtain spheroids, AggreWell™800 microwell plates (STEMCELL Technologies, Canada) were treated with Anti-Adherence Rinsing Solution (STEMCELL Technologies, Canada) for 10 minutes and then washed with 1X PBS. Cells were dissociated using 0.25 % trypsin solution with EDTA and seeded at a rate of 3×10^6 cells per well in 2 ml of growth medium. Half the volume of the medium was changed the next day. On day 3, spheroids were washed out of micro-well plates and transferred to 10 cm dishes were cultured in a shaker incubator at 37 °C, 5 % CO_2 and constant agitation at 80 rpm, changing the medium every 3–4 days.

Assessment of cytotoxic activity of VV-GMCSF-Lact against U-87 MG cells

U-87 MG cells were seeded onto a 96-well plate at a concentration of 4×10^3 cells per well in 100 µL of phenol red-free Opti-MEM medium and incubated at 37 °C in a 5 % CO_2 atmosphere. After 24 h, VV-GMCSF-Lact was added to the cells. The multiplicity of virus infection ranged from 0.0012 to 10 PFU per cell. Cells were incubated with the virus for 72 h. Control cells were incubated under the same conditions without the addition of viral preparation. After incubation, 10 µL of Deep Blue Viability Kit reagent (BioLegend, USA) was added to the wells and incubated for 4 h at 37 °C. The optical density of the solution was measured at a wavelength of 570 nm (reference value – 620 nm) using an Apollo LB 912 spectrophotometer (Berthold Technologies, Germany). Cell viability was determined relative to the viability of control cells (100 %) ± SD following three independent experiments.

Assessment of cytotoxic activity of VV-GMCSF-Lact against 3D-cultured human glioblastoma U-87 MG cells

3D cultures of U-87 MG cells were placed in wells of a 96-well plate at 10 cells per well and cultured in 100 µL of complete DMEM/F12 culture medium. After 24 h, the medium was removed and replaced with 50 µl of virus suspension with a virus titre of 3.3×10^7 PFU/ml. 50 µl of 1 mM TRIS HCl (pH = 8.5) was added to control wells. The plate was centrifuged for 20 min at 500 rpm to sediment the virus. The cells were then incubated in a CO_2 incubator at 37 °C for 20 min, after which 50 µL of complete DMEM/F12 culture medium was added. The cytotoxic effects of the virus were analyzed at 24 and 72 hours and on day *7* after infection of cells.

Microscopy

3D cell culture samples after exposure to the virus were analysed *in vivo*, in transmitted light and in the FITC green channel using a Nicon Eclipse Ti microscope (Tokyo, Japan).

Cytofluorimetric analysis

Cell samples after virus exposure were dissociated enzymatically using Accumax reagent (Stemcell Technologies,

Canada) and analysed with a BD FACS Canto II flow cytometer (Becton Dickinson, USA) using BD Pharmigen FITC Annexin V Apoptosis Detection Kit I, according to the manufacturer's instructions with minor modifications: only one dye, propidium iodide (PI), was used, as Annexin V – FITC should be analysed in the green channel already occupied due to the transduction of U-87 MG cells. Cytofluorimetry results were analyzed in FlowJo™ v. 10 Software (Becton Dickinson, USA).

VV-GMCSF-Lact virus titration

Green monkey kidney 4647 cells were seeded in a 12-well plate containing 300,000 cells per well, in 2 ml of complete DMEM medium with 10 % fetal serum (FBS) and incubated at 37 °C in a 5 % CO_2 atmosphere until 90 % monolayer formation. 10-fold dilutions of the viral suspension in DMEM medium were prepared. Growth medium was removed from the wells of the plate and 100 µl of the appropriate dilution of virus was added to the centre of each well. The plate with cells was incubated at 37 °C in a CO_2 incubator for 1 hour. Then 2 ml of minimum essential medium (DMEM + 2 % FBS) was added to the wells and the plate was left at 37 °C in a CO_2 incubator for 48 hours. Afterwards, the minimum essential medium was removed, 1 ml of crystal violet solution was added to the wells with cells and left for 20 min at room temperature. Then the crystalline violet solution was removed and the plaques were counted.

Statistical analysis

Quantitative variables are presented as mean ± standard deviation (SD). Each experiment was repeated at least three times. Statistical analysis was performed using GraphPad 6.01 (GraphPad Software, USA). Two-factor analysis of variance was used to compare more than two data sets. Differences were considered statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

U-87 MG human glioblastoma cells were transduced with a lentiviral construct carrying a *GFP* reporter gene, as it is planned to use these cells for co-culture with human cerebral organoids in the future. The cytotoxicity of the virus against U-87 MG cells was determined by cell proliferative activity using resazurin as an indicator. The degree of reduction of resazurin and production of resorufin is proportional to the number of metabolically active cells. The IC50 (half maximal inhibitory concentration) measured in CompuSyn software was 0.024 PFU/cell, indicating a higher sensitivity of this cell culture to VV-GMCSF-Lact virus compared to the non-transduced lentivirus U-87 MG cell culture [4].

Formed 3D cultures of U-87 MG were incubated with VV-GMCSF-Lact, which had a titer of 3.3×10^7 PFU/ml. This amount of viral particles is comparable to that of intratumoural injection into the body of a laboratory animal in preclinical trials [3]. The 3D-cultured cells were further analyzed 24 and 72 h and 7 days after cell infection. It has been revealed that replication of some oncolytic viruses can increase as early as 24 h after infection of tumour cells, however, after 72 h it starts to decrease [5, 6].

Microscopic analysis of the samples was performed both in transmitted light and using a fluorescent filter for fluorescein isothiocyanate (FITC). In our study, oncolytic effect of VV-GMCSF-Lact was observed on a 3D culture of U-87 MG cells (Fig. 1).

The structure of 3D-cultures is destroyed and the amount of cellular debris increases with extending incubation time with virus ("3D-cultures with virus_24 hours", "3D-cultures with virus_72 hours", "3D-cultures with virus_7 days"), whereas in control wells 3D cultures ("Control 3D-cultures_24 hours", "Control 3D-cultures_72 hours", "Control 3D-cultures_72 hours", "Control 3D-cultures_7 days") remain unchanged, preserving clear outlines and smooth edges. The intensity of cell death reactions was assessed by the interaction of cells with propidium iodide (PI), a marker of necrosis. Unfortunately, no statistically significant difference was observed between groups in the number of stained cells in the PE channel, possibly due to the presence of necrotic core in 3D cultures. As tumour spheroid reaches a diameter of more than 500 µm, it is known that the spheroid usually exhibits a three-layer concentric structure including an outer layer of proliferating cells, a middle layer of quiescent cells and a central necrotic zone, with each region at different stages of the cell cycle [7]. The complexity of this multilayered structure may be caused by a lack of oxygen and nutrients, which is not observed in 2D tumour cell cultures. Drugs, soluble metabolites, as well as oxygen concentration and pH are known to exist as a gradient within the tumour: peripheral cells closer to blood vessels have greater access to soluble components and oxygen, which decreases as it diffuses through the extracellular matrix to the tumour core. The concentration gradients of growth factors, nutrients, and metabolites create intratumoural heterogeneity and affect signalling in the microenvironment, including cell function, proliferation, morphogenesis, and chemotaxis [8]. A concentration gradient, from a pharmacokinetic point of view, limits the penetration of drugs into the tumour and the attainment of a dosage sufficient to exert therapeutic effects on all cancer cells.

Using cytofluorimetric analysis, a statistically significant increase in side scatter intensity, i.e. cell granularity of 3D-cultured cells at time points 24 and 72 h (Fig. 2) was observed, which indirectly attributes to active replication of the virus in the cells. For instance, it was previously revealed that cell populations with higher SSC (side scatter) intensity had more inclusions, more organelles and virosomes (viral factories) [9, 10].

To assess the replication efficiency of VV-GMCSF-Lact in 3D-cultured human glioblastoma cells, the virus titre was determined on day 7 after cell infection [11]. The virus was revealed to replicate efficiently in spheroid cells; the titer was 0.44 PFU/cell, which is ≈18-fold higher than the IC50 for the corresponding adherent cultures, further confirming the literature data about the resistance of 3D cultures compared with adherent cells when testing the cytotoxic-

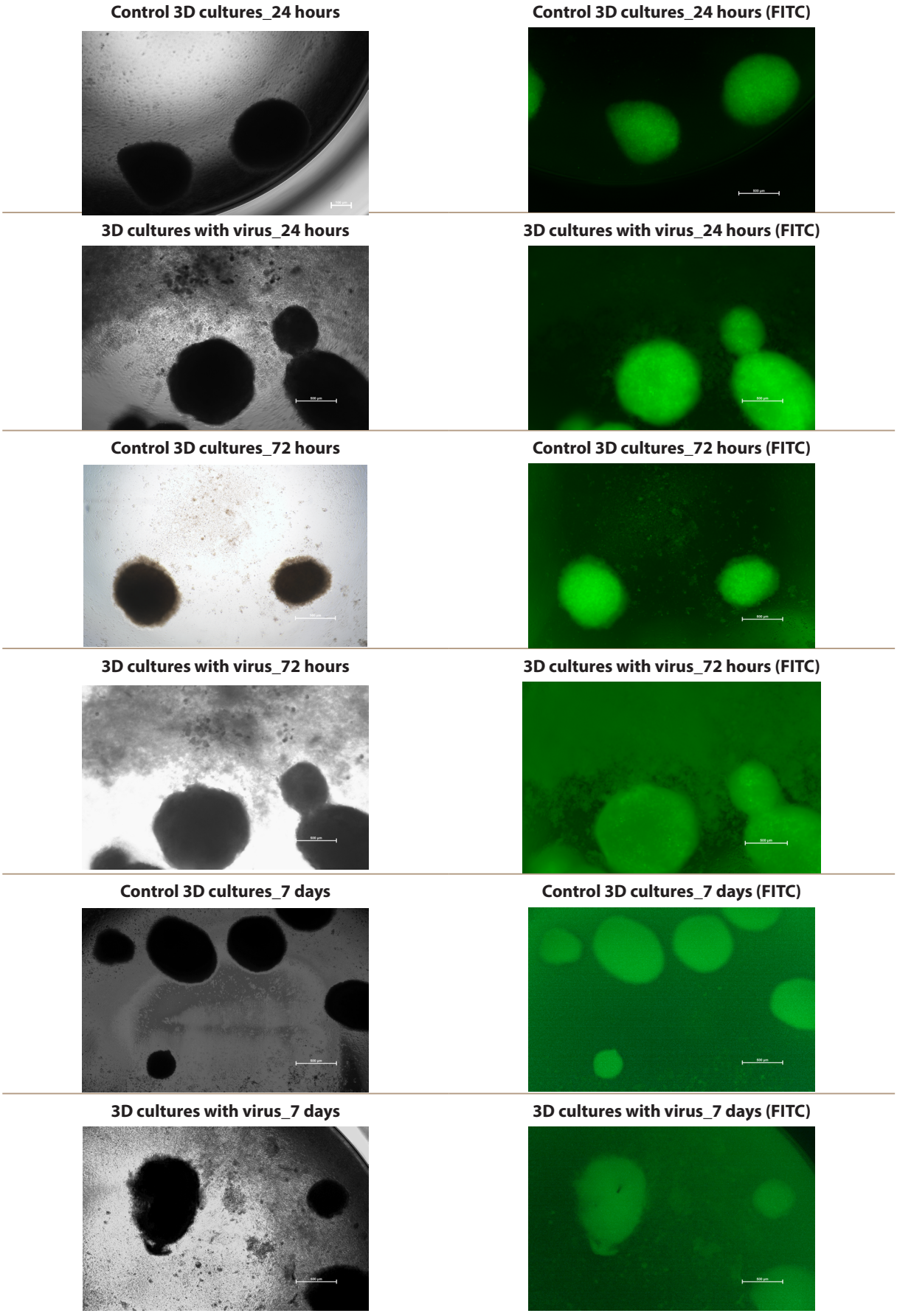

FIG. 1.

Microscopic image of 3D-cultured cells treated with oncolytic virus VV-GMCSF-Lact and untreated control 3D-cultured cells; microscope magnification × 40

ity of anti-tumour drugs [12]. It is likely that virus replication occurs in the outer cell layer of the spheroid, but further experiments are needed to pinpoint the exact localization of this process.

Consequently, cells cultured in larger three-dimensional aggregates mimic the *in vivo* state by being in different proliferative states based on nutrient access that is limited by a concentration gradient. Whilst 2D cultures are still predominantly used for drug development due to their simplicity and compatibility with screening platforms, 3D culture systems have numerous advantages over 2D cell culture. Specifically, 3D cell culture models more accurately reflect the pathophysiological microenvironment that allows tumour cells to aggregate, proliferate and exhibit phenotypes as they do within the body [13]. Complex cellular interactions between other cells and the three-dimensional matrix are crucial for preserving the structure, function and motility of tumour cells. Since cell migration occurs in three dimensions, the matrix provides a topology that mimics the three-dimensional architecture of tissue, allowing cells to attach and interact with the environment.

CONCLUSION

3D cultures are a more relevant model for testing anti-tumour drugs compared to adherent models. Additionally, the move to 3D preclinical models has become more attractive as improvements in tissue engi-

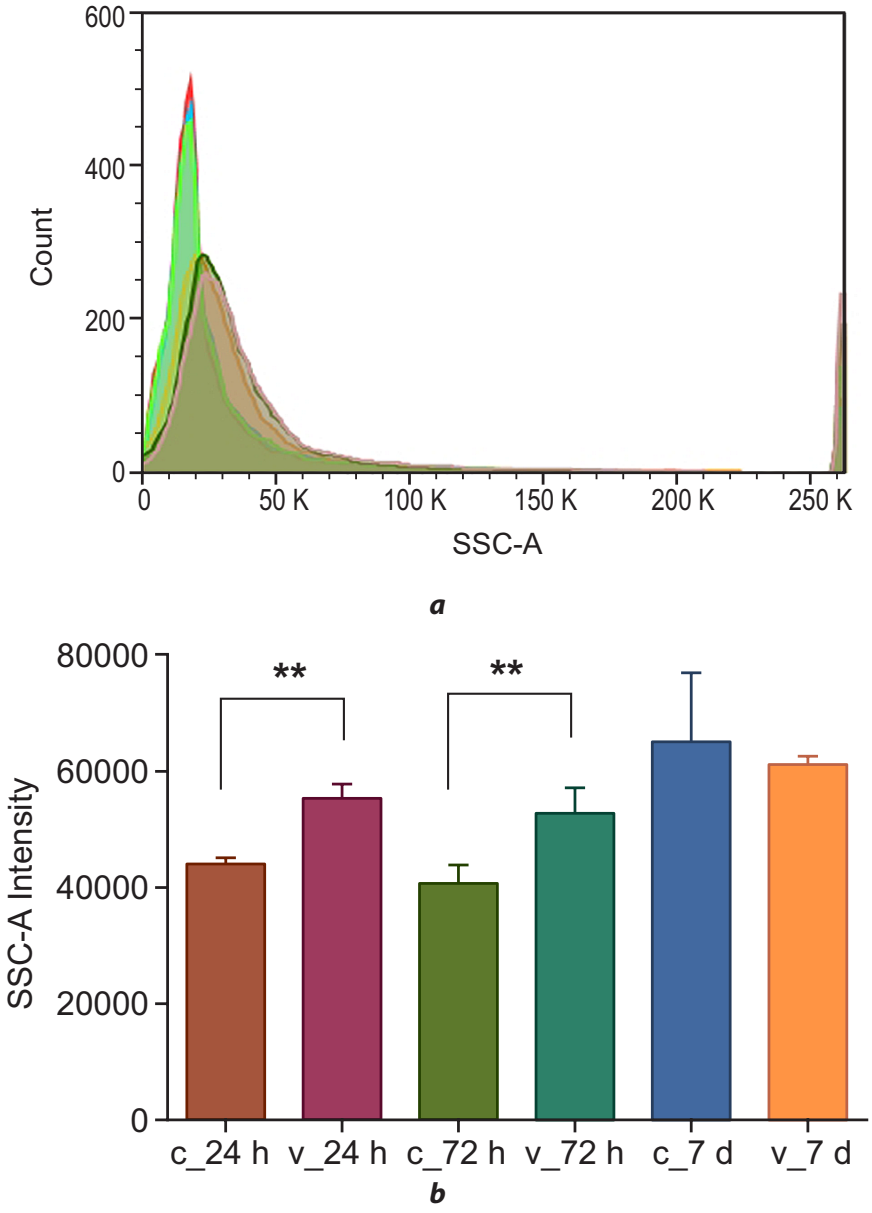

FIG. 2.

a *– distribution of 3D-cultured cells by side scatter intensity; flow cytometry;* ***b*** *– average side scatter intensity of 3D-cultured cells treated with virus (v_24 h, v_72 h, v_7 d) compared to control group cells (c_24 h, c_72 h, c_7 d)*

neering technologies have made 3D cell culture more adaptable and adjustable to microenvironmental factors so as to better reflect the functional pathology of tumours *in vivo*. The use of 3D cultures allows, among other things, to assess the ability of oncolytic drugs to penetrate the tumour and affect its internal structures. Meanwhile, the formation of a necrotic tumour core complicates the analysis of the oncolytic action of the virus by cytometry. Therefore, several analytical methods should be applied to assess the anti-tumour efficacy of therapeutic agents.

Subsequently, it is planned to use not only 3D cultures of human glioblastoma, but also chimeric cerebral organoids obtained by co-culturing tumour cells and human cerebral organoids to test the cytotoxic effect of VV-GMCSF-Lact [14]. Such a cell model allows to recreate three-dimensional cytoarchitectonics of some parts of the brain, which opens unique opportunities to study the interaction between tumour cells and brain cells, as well as the effect of oncolytic drug on healthy nervous tissue and glial tumour microenvironment [15].

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Maya A. Dymova – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: maya.a.rot@gmail.com, https://orcid.org/0000-0002-7281-9096

Tatyana A. Shnaider – Junior Research Officer at the Department of Genomic Mechanisms of Ontogenesis, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, e-mail: shnayder.t@yandex.ru, https://orcid.org/0000-0002-7452-8372

Sophia A. Chechetkina – student, Novosibirsk State University, e-mail: s.tschetschetkina@gmail.com; https://orcid.org/0000-0003-0656-5806

Gleb O. Petrov – Senior Clinical Research Assistant at the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: petrov.olegovich@gmail.com, https://orcid.org/0000-0002-2924-5920

Daria O. Malysheva – Clinical Research Assistant at the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: d.malysheva@g.nsu.ru, https://orcid.org/0009-0005-1210-1815

Danil V. Drokov – student, Novosibirsk State University, e-mail: d.drokov@g.nsu.ru, https://orcid.org/0009-0001-7343-4438

Alisa B. Ageenko – Engineer of the First Category at the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: a.ageenko@alumni.nsu.ru, https://orcid.org/0000-0002-6909-7301

Natalia S. Vasileva – Junior Research Officer at the Laboratory of Genomic Editing, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: nataly_vas@bk.ru, https://orcid.org/0000-0002-0392-794X

Vladimir A. Richter – Head of the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: richter@niboch.nsc.ru, https://orcid.org/0000-0001-5849-5892

Elena V. Kuligina – Cand. Sc. (Biol.), Senior Research Officer at the Laboratory of Biotechnology, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, e-mail: kuligina@niboch.nsc.ru, https://orcid.org/0000-0003-3145-1878

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OPHTHALMOLOGY

A CLINICAL CASE OF A COMBINED METHOD FOR CORRECTING POSTKERATOPLASTIC ASTIGMATISM OF A HIGH DEGREE IN A PATIENT WITH CATARACT

Sinitsyn M.V.,
Pozdeyeva N.A.

Cheboksary Branch of the S. Fyodorov Eye Microsurgery Federal State Institution (Traktorostroiteley ave. 10, Cheboksary 428028, Russian Federation)

Corresponding author:
Maxim V. Sinitsyn,
e-mail: mntksinicin@mail.ru

ABSTRACT

Background. *Performing penetrating keratoplasty in 100 % of cases leads to the occurrence of induced postkeratoplasty astigmatism, which can be more than 12.0 D. If cataracts occur in this category of patients, performing cataract phacoemulsification with implantation of a toric intraocular lens (tIOL) does not completely solve the problem. The use of the method of implantation of intrastromal corneal segments at stage I before cataract phacoemulsification makes it possible to reduce the degree of postkeratoplasty astigmatism and create optimal conditions for additional correction of residual postkeratoplasty astigmatism due to the implantation of tIOL during cataract phacoemulsification.*

The aim of the study. *To analyze the clinical and functional indicators of correction of regular high-grade postkeratoplasty astigmatism in a patient with cataracts using a combined method, including first implantation of intrastromal corneal segments and subsequent cataract phacoemulsification with implantation of toric intraocular lens using the example of a clinical case.*

Material and methods. *A 55-year-old patient with cataracts, who had a history of undergoing penetrating keratoplasty, contacted us. According to the keratotopogram data, a regular postkeratoplasty astigmatism of 18.68 D was diagnosed. The patient underwent a combined method. At stage I, intrastromal corneal segments were implanted into the corneal graft, then after 6 months stage II was performed – cataract phacoemulsification with implantation of tIOL.*

Results. *Six months after intrastromal corneal segments implantation, the patient's keratometric data stabilized, and corneal astigmatism decreased to 8.98 D. Then the patient underwent cataract phacoemulsification with tIOL implantation. After 1 month, the spherical component of refraction was 0.5 D, the cylindrical component of refraction was –0.5 D, visual acuity increased to 1.0.*

Conclusion. *A combined method for correcting regular high-grade postkeratoplasty astigmatism in a patient with cataracts showed high refractive results, stability and safety in the long-term postoperative period.*

Key words: *postkeratoplastic astigmatism, intrastromal corneal segments, cataract*

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КЛИНИЧЕСКИЙ СЛУЧАЙ КОМБИНИРОВАННОГО СПОСОБА КОРРЕКЦИИ ПОСТКЕРАТОПЛАСТИЧЕСКОГО АСТИГМАТИЗМА ВЫСОКОЙ СТЕПЕНИ У ПАЦИЕНТА С КАТАРАКТОЙ

Синицын М.В.,
Поздеева Н.А.

Чебоксарский филиал ФГАУ «НМИЦ «МНТК «Микрохирургия глаза» имени академика С.Н. Фёдорова» Минздрава России (428028, г. Чебоксары, просп. Тракторостроителей, 10, Россия)

Автор, ответственный за переписку:
Синицын Максим Владимирович,
e-mail: mntksinicin@mail.ru

РЕЗЮМЕ

Введение. *Выполнение сквозной кератопластики (СКП) приводит в 100 % случаев к возникновению индуцированного посткератопластического астигматизма (ПА), который может быть более 12 dптр. При возникновении у данной категории пациентов катаракты выполнение факоэмульсификации катаракты (ФЭК) с имплантацией торической интраокулярной линзы (тИОЛ) не позволяет полностью решить проблему. Применение метода имплантации интрастромальных роговичных сегментов (ИРС) на I этапе до ФЭК позволяет снизить степень ПА и создать оптимальные условия для докоррекции остаточного ПА за счёт имплантации тИОЛ во время ФЭК.*

Цель исследования. *Провести анализ клинико-функциональных показателей коррекции регулярного посткератопластического астигматизма высокой степени у пациента с катарактой комбинированным способом, включающим вначале имплантацию интрастромальных роговичных сегментов и последующее выполнение факоэмульсификации катаракты с имплантацией торической интраокулярной линзы, на примере клинического случая.*

Материал и методы. *К нам обратился пациент 55 лет с катарактой, в анамнезе у которого была выполнена сквозная кератопластика. По данным кератотопограммы был диагностирован регулярный ПА 18,68 dптр. Пациенту был выполнен комбинированный метод. На I этапе в роговичный трансплантат были имплантированы ИРС, затем через 6 мес. был выполнен II этап – ФЭК с имплантацией тИОЛ.*

Результаты. *Через 6 мес. после имплантации ИРС у пациента произошла стабилизация кератометрических данных, роговичный астигматизм снизился до 8,98 dптр. Затем пациенту была выполнена ФЭК с имплантацией тИОЛ. Через 1 мес. сферический компонент рефракции составил 0,5 dптр, цилиндрический компонент рефракции –0,5 dптр, острота зрения повысилась до 1,0.*

Заключение. *Комбинированный способ коррекции регулярного ПА высокой степени у пациента с катарактой показал высокий рефракционный результат, стабильность и безопасность в отдалённом послеоперационном периоде.*

Ключевые слова: *посткератопластический астигматизм, интрастромальные роговичные сегменты, катаракта*

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RELEVANCE

The occurrence of cataract in patients after penetrating keratoplasty (PK) reduces their visual acuity and requires its surgical treatment [1]. Conversely, an induced postkeratoplasty astigmatism (PA) is diagnosed after PK in every case, which can be high-grade and irregular [2]. This in turn leads to an increase in corneal aberrations, especially of higher orders, reducing visual acuity and leading to patient dissatisfaction with the optical outcome of surgery [3]. Performing cataract phacoemulsification (CPE) with toric intraocular lens (tIOL) implantation allows to simultaneously get rid of cataract and compensate corneal astigmatism in its regular form [4, 5]. This technique, however, is limited to the toric component of manufactured tIOLs up to and including 12 D. In summary, correction of regular PA greater than 12 D in a cataract patient requires an additional method of correction. Currently, implantation of intrastromal corneal ring segments (ICRS) into the corneal graft to increase corneal graft regularity and reduce PA is gaining popularity as a result of the lack of PA regression years after surgery compared to refractive laser surgery [6, 7]. In view of the above, the ICRS implantation method can be used in the combined treatment of such cataract patients with PA greater than 12 D.

THE AIM OF THE STUDY

To analyse the clinical and functional indices in the correction of regular high-grade postkeratoplasty astigmatism in a patient with cataract by a combined method including first implantation of intrastromal corneal ring segments using femtosecond laser followed by cataract phacoemulsification with toric intraocular lens implantation based on the example of a clinical case.

MATERIAL AND METHODS

Patient N., 55 years old, addressed the branch of the S.N. Fedorov Eye Microsurgery Centre of the Russian Ministry of Health with complaints of low vision and fog in front of the left eye for the last 2 years. Ophthalmological history: in 2002, a penetrating keratoplasty (PK) was performed on the right eye concerning stage IV keratoconus. On admission, uncorrected visual acuity (UCVA) was 0.03 i/c (incorrigible), intraocular pressure was 15 mmHg according to Maklakov, and the anteroposterior axis of the eye was 23.54 mm. At biomicroscopy, the corneal graft was transparent, had a diameter of 8.0 mm; the anterior chamber had a medium depth; pupil – 3.5 mm, photoreaction of III degree; iris was quiet, without structural changes; posterior capsular opacities were visualised in the crystalline lens, the underlying media were not clearly seen due to lens opacity. According to the keratotopogram on the TMS-4 device (Tomey, Japan), a regular high-grade PA of 18.68 D was visualised (Fig. 1).

According to the results of corneal optical coherence tomography (OCT) using an OCT Casia 2 (Tomey, Germany), the minimum thickness of the corneal graft in the centre was 457 μm. Endothelial cell density (ECD) measured on a Confoscan-4 device (Nidek, Japan) was 1925 cells/mm^2. According to the electrophysiological study on the Diopsys device (NOVA, USA), the lability of the optic nerve of the left eye was within the normal range. According to ultrasound B-scan on a Tomey UD-8000 (Tomey, Germany), the sheaths are adherent. According to the results of the examination, the diagnosis for the left eye was high-grade regular postkeratoplasty astigmatism, condition after penetrating keratoplasty, posterior capsular cataract.

Correction of regular PA was performed in 2 stages. The first stage was the implantation of two ICRS in the penetrating corneal graft using femtosecond laser

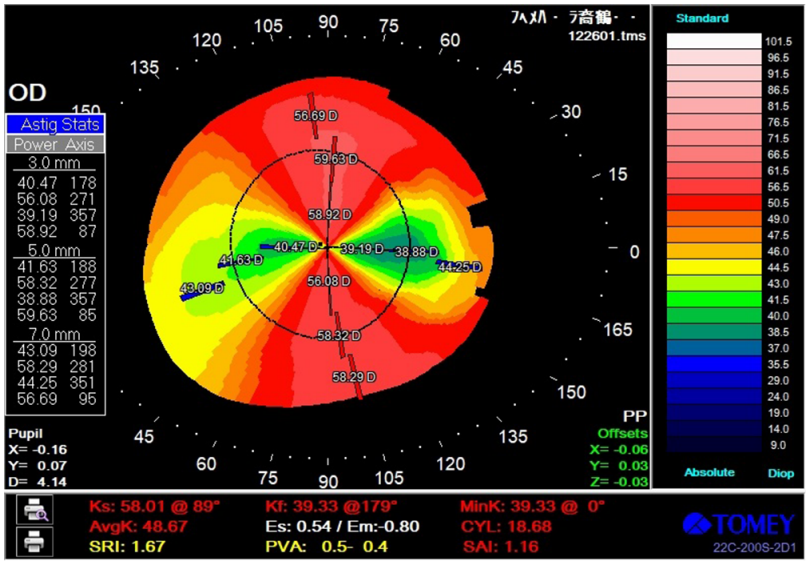

FIG. 1.

Keratotopogram of patient N. with induced regular high-grade postkeratoplasty astigmatism

(FSL) to reduce PA less than 12 D. Two identical ICRS (LLC Research and Experimental Production of Eye Microsurgery, Russia) made of polymethylmethacrylate were implanted in the patient, having the shape of a hemisphere on the cross section; inner radius – 5.0 mm, outer radius – 6.2 mm, height – 350 μm, arc length – 90°. ICRS indicators for implantation in the corneal graft were calculated according to the existing nomograms [8]. Then in the second stage, 6 months after stabilisation of clinical and functional parameters, CPE with implantation of tIOL for additional correction of residual PA was performed. This sequence was chosen not only to reduce corneal astigmatism (RA) at stage 1 by implanting ICRS in the corneal graft, but mainly to create optimal conditions for accurate tIOL calculation and centration at stage 2 by increasing the sphericity and regularity of the corneal graft.

ICRS implantation into a penetrating corneal graft was performed using the Russian 1 MHz FemtoViaisum FSL (Troitsk, Russia). Prior to surgery, the patient was marked the location of the intrastromal tunnel symmetrically relative to the patient's visual axis, which was determined by the Purkinje light reflex when the patient looked at the fixation mark of the operating microscope. The surgery involved two phases. In the first stage, an intrastromal tunnel was formed in the corneal graft with an inner resection diameter of 5.0 mm and an outer diameter of 6.2 mm, at a depth of 385 μm, using FSL. At the second stage, two ICRS with a height of 350 μm, with an arc length of 90° were implanted through the entrance vertical incision into the formed intrastromal tunnel and placed parallel to the strong axis of the PA according to the keratotopogram in order to flatten it between the implanted ICRS.

Six months after ICRS implantation in the penetrating corneal graft, the patient underwent CPE with implantation of tIOL AT Torbi 709M (Carl Zeiss, Germany) with an optical power of 16 D with a toric component of 8.0 D for additional correction of residual corneal astigmatism. Keratotopographic data, optical biometry data and online IOL calculators (Kane Formula, Barrett Universal II Formula) were used to calculate the optical power of the IOL. Prior to surgery, the patient was marked behind a slit lamp to mark the horizontal axis of the corneal graft. During preoperative pre-surgical preparation, the patient received instillation of a non-steroidal anti-inflammatory drug, and on the morning of the day of surgery, he additionally received mydriatic instillation of one drop three times 30 min before surgery. CPE with tIOL implantation was performed using the Infinity device (Alcon, USA) according to the standard technique. Using a Mendes ring on the operating table, the patient was marked with a strong corneal graft keratometry axis, along which a 1.8–2.2 mm long main tunnel incision was made, with paracenteses located at 3 and 9 o'clock. The depth of the anterior chamber was controlled at the stage of capsulorhexis by injecting viscoelastic into it. Capsulorhexis was performed with a curved insulin needle. Removal of the lens nucleus was performed using a phacoemulsifier. The cortical masses and epinuclius were removed using a coaxial irrigation-aspiration handpiece. tIOL was implanted with an injector, and then the marks of the cylindrical component of the tIOL were centred on the marks placed on the corneal graft before surgery, corresponding to the strong axis of the PA, according to the keratotopogram data. At the end of surgery, viscoelastic was flushed out of the anterior chamber using a Simcoe cannula. The main incision and paracentesis were sealed with sterile saline solution. Subconjunctival injection of antibiotic and corticosteroid was performed at the completion of surgery.

Apart from the standard methods of examination, the patient underwent: optical biometry with determination of eye length, anterior chamber depth, lens thickness on A-Scan Plus (Stormoff NRW Gmbh, Germany); keratotopography using keratotopograph Tomey 4 (Tomey, Japan); calculation of ECD on Confoscan-4 device (Nidek, Japan); keratopachymetry using OCT Casia 2 (Tomey, Germany); assessment of visco-elastic properties of the corneal graft using ORA device (Reichert, USA); measurement of protein flux and number of cells in the anterior chamber moisture using FS-2000 device (Kowa, Japan). The follow-up period after CPE was 6 months.

RESULTS

No intra- and postoperative complications were noted when performing IRS implantation into a penetrating corneal graft using FSL. At biomicroscopy on the first day after surgery, the penetrating corneal graft was transparent, ICRS were centred, posterior capsular opacities were visualised in the lens, and the underlying media were not clearly visible due to lens opacities.

At the examination on the day after ICRS implantation, the patient's UCVA increased by 0.02, corrected visual acuity (CVA) by 0.07, and these parameters did not change again during 6 months (Table 1).

The spherical component of refraction (SCR) could not be measured due to lens opacity. Corneal astigmatism (CA) decreased by 10.58 D; mean keratometry value (Km.) – by 1.42 D and did not change any more; corneal surface regularity index (SRI) – by 0.39; corneal surface asymmetry index (SAI) – by 0.3; corneal resistance factor (CRF) increased by 0.5 mmHg; corneal hysteresis (CH) – by 0.3 mmHg.

At 1 month after the surgery, CA increased by 0.45 D, SRI decreased by another 0.06, SAI by another 0.09, CRF increased by another 0.2 mmHg, and CH by 0.4 mmHg.

At 6 months after the surgery, CA increased by another 0.43 D, SRI decreased by another 0.12, SAI – by another 0.25, CRF increased by another 0.5 mmHg, CH – by 0.3 mmHg [2].

Thus, ICRS implantation into the corneal graft not only significantly decreased PA but also increased the sphericity and regularity of the corneal graft, as evidenced by decreased CA, SRI and SAI indices. This, in turn, has provided optimal conditions for complete additional correction of residual CA in CPE with IOL implantation by increasing the accuracy of IOL measurement and centring, as the keratometry value along the main meridians began to change

TABLE 1

DATA OF CLINICAL AND FUNCTIONAL PARAMETERS BEFORE AND AT DIFFERENT FOLLOW-UP PERIODS AFTER IMPLANTATION OF INTRASTROMAL CORNEAL RING SEGMENTS IN THE PENETRATING CORNEAL GRAFT WITH FEMTOSECOND LASER APPLICATION

Indicators	Before the surgery	Day 1 after the surgery	1 month after the surgery	6 months after the surgery
UCVA	0.03	0.05	0.05	0.05
CVA	0.03	0.1	0.1	0.1
SCR, D	unmeasured	unmeasured	unmeasured	unmeasured
CA, D	–18.68	–8.1	–8.55	–8.98
Km., D	48.67	47.25	47.25	47.25
SRI	1.67	1.28	1.22	1.1
SAI	1.16	0.84	0.75	0.5
KG, mmHg	8.1	8.4	8.8	9.1
CRF, mmHg	8.4	8.9	9.1	9.6
Penetrating corneal graft pachymetry minimum value in the centre, μm	457	462	459	458
ECD, cells/mm^2	1925	1925	1921	1906
Protein flux in anterior chamber moisture, f/m (focus microscope)	2.3	3.15	2.3	2.3
Number of cells in the anterior chamber moisture, cells/mm^3	1.55	2.3	1.6	1.63

Note. SCR – spherical component of refraction; Km. – mean keratometry value; SRI – Surface Regularity Index; SAI – Surface Asymmetry Index; CH – corneal hysteresis; CRF – corneal resistance factor.

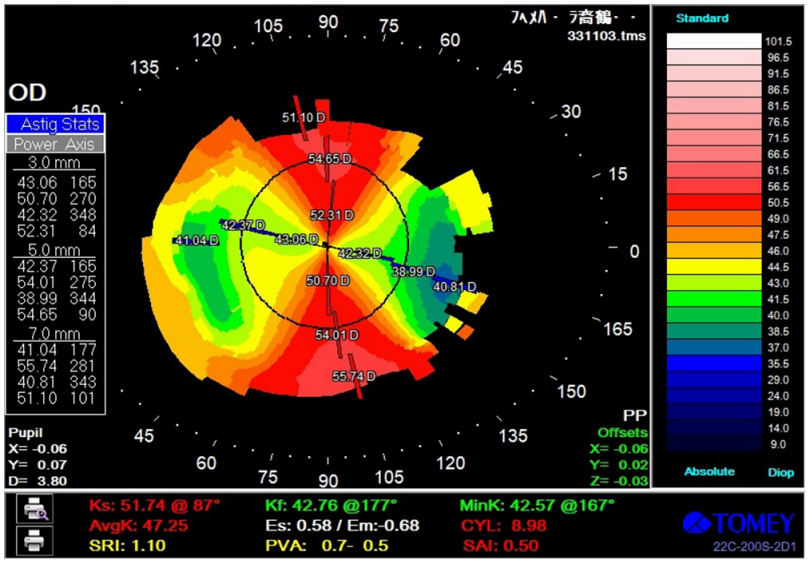

FIG. 2.

Keratotopogram of patient N. 6 months after implantation of intrastromal corneal ring segments in the through corneal graft using femtosecond laser: there is an increase in regularity and sphericity of the corneal graft with a decrease in the degree of corneal astigmatism by 9.7 D

more symmetrically, which was confirmed by the achievement of normal values of the SAI index. In contrast, ICRS implanted in the corneal graft resulted in an additional "stiffness frame" in the corneal graft, which increased its biomechanical properties. According to literature data, it is the increase of biomechanical properties of the corne-

al graft that allows to preserve the obtained refractive result in the remote postoperative period, in contrast to refractive laser surgeries, in which biomechanical properties of the corneal graft weaken when its thickness decreases, which in turn causes regression of the refractive result over the years [7].

The correct ICRS position was confirmed by OCT data of the corneal graft (Fig. 3).

In the postoperative period, corneal OCT data showed no change in corneal graft thickness at the centre.

The loss of ECD 6 months after ICRS implantation in an end-to-end corneal graft using CPE was 1.0 %, which did not exceed the physiological loss. According to the FC-2000 device, the cell count and protein flux in the anterior chamber moisture on the day after surgery increased slightly, but did not exceed the limits of normal. Laser tindalmetry values reached preoperative values within 1 month after surgery and did not increase again.

Six months after ICRS implantation in the corneal graft, the patient underwent CPE with tIOL implantation for a single-stage additional correction of residual corneal astigmatism.

No intra- and postoperative complications were observed. The patient noted subjectively a significant increase in visual acuity after surgery. On biomicroscopy, the optical media were clear and the two implanted ICRS and tIOL were centred (Fig. 4).

On examination the day after CPE, the UCVA increased by 0.85 and the CVA increased by 0.8. CA decreased by –0.48 D. The SCR was 1.0 D, and the cylindrical refractive component (CRC) was –1.0 D (Table 2).

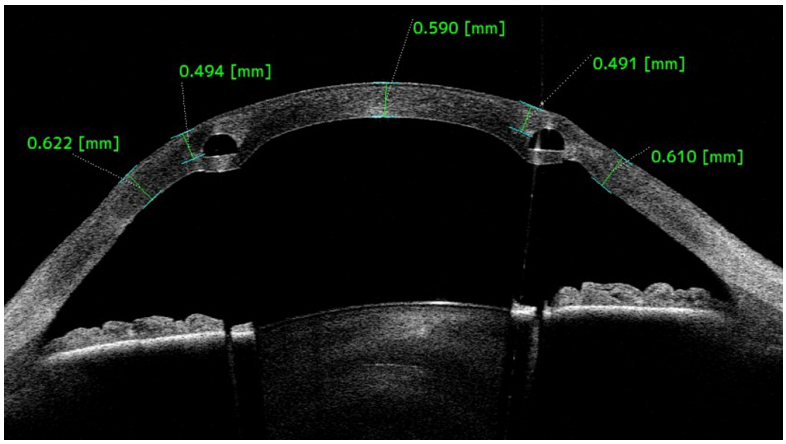

FIG. 3.

Optical coherence tomography of patient N.'s penetrating corneal graft after implantation of intrastromal corneal segments using femtosecond laser: the profile of intrastromal corneal ring segments located at a depth of 385 μm is visualised

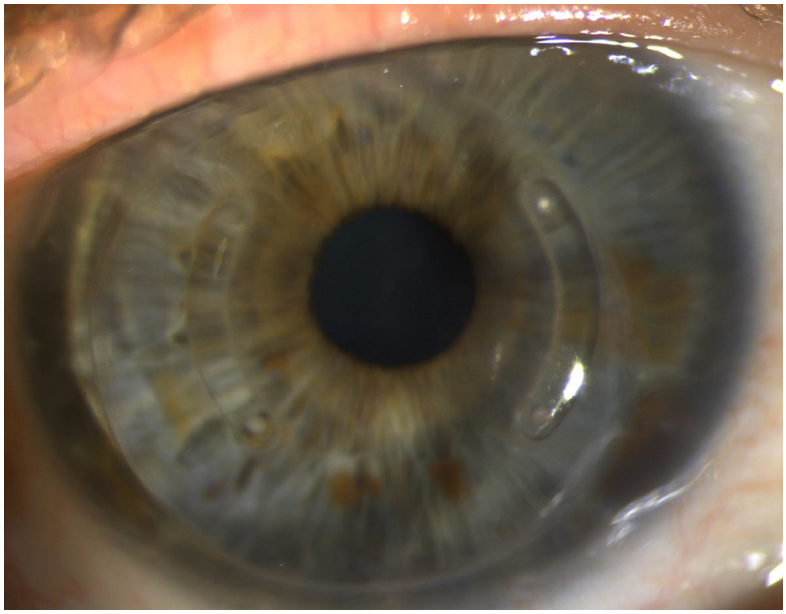

FIG. 4.

Eye appearance of patient N. on the next day after cataract phacoemulsification with toric IOL implantation after earlier implantation of intrastromal corneal ring segments into the corneal graft using femtosecond laser: symmetrically located intrastromal corneal ring segments relative to the strong vertical meridian of the corneal graft are visualised, toric IOL in the pupil projection is visualised

TABLE 2

DATA OF CLINICAL AND FUNCTIONAL INDICATORS BEFORE AND AT DIFFERENT TERMS AFTER CATARACT PHACOEMULSIFICATION WITH TORIC IOL IMPLANTATION IN A PATIENT AFTER EARLIER ICRS IMPLANTATION IN THE CORNEAL GRAFT WITH FEMTOSECOND LASER APPLICATION

Indicators	Before the surgery	Day 1 after the surgery	1 month after the surgery	6 months after the surgery
UCVA	0.05	0.9	1.0	1.0
CVA	0.1	0.9	1.0	1.0
CA, D	–8.98	–8.5	–8.25	–8.25
SCR, D	unmeasured	1.0	0.5	0.5
CRC, D	unmeasured	–1.0	–0.5	–0.5
ECD, cells/mm^2	1906	1801	1797	1781
Protein flux in anterior chamber moisture, f/m (focus microscope)	2.3	12.4	2.2	2.3
Number of cells in the anterior chamber moisture, cells/mm^3	1.63	11.1	1.68	1.65

At 1 month after surgery, the UCVA and CVA increased by another 0.1; CA decreased by another –0.25 D, SCR – by 0.5 D, CRC – by –0.5 D, and these indices remained unchanged.

In the surgical treatment of cataract in patients after PK, the initial preoperative PEC, as well as the degree of its loss after CPE, is of great importance. In the described clinical case, the preoperative ECD value of 1906 cells/mm^2 is sufficient for CPE. When ECD was measured on the day after CPE, it was observed to decrease by 5.5 % to a value of 1801 cells/mm^2, which is almost 3 times higher than its critical value of 500–700 cells/mm^2 [9]. Thus, the surgery was safe in terms of the risk of graft-versus-host disease. According to the literature, the use of modern viscoelastics during cataract surgery in patients after PK results in a loss of ECD in the range of 5–8 %, which is consistent with the results of our study [10]. ECD loss increased by another 1.1 % by 6 months postoperatively, which does not exceed the physiological loss of 2.5 % in the six months after PK [11].

On the day after CPE, when protein flux was counted in the anterior chamber using the FS-2000 device, a 5.4-fold increase was observed, and the number of cells in the anterior chamber moisture was 6.8-fold. One month after CPE, the values of these indices corresponded to preoperative values and did not change any more.

CONCLUSION

The combined method of correction of regular high-grade PA in a patient with cataract by ICRS implantation in the corneal graft followed by CPE with tIOL implantation showed a high refractive result, stability and safety in the distant postoperative period.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Maxim V. Sinitsyn – Cand. Sc. (Med), Head of the Pediatric Department, Cheboksary Branch of the S. Fyodorov Eye Microsurgery Federal State Institution, e-mail: mntksinicin@mail.ru, https://orcid.org/0000-0002-7285-1782

Nadezhda A. Pozdeyeva – Dr. Sc. (Med.), Director, Cheboksary Branch of the S. Fyodorov Eye Microsurgery Federal State Institution, e-mail: mntksinicin@mail.ru, https://orcid.org/0000-0003-3637-3645

PEDIATRICS

ATTITUDE AND AWARENESS OF INDIAN PARENTS FROM KERALA STATE TOWARDS CHILDREN'S VACCINATION AT THE COVID-19 PANDEMIC BACKGROUND

Aaromal Ajitha Sureshkumar [1], Novikova E.A. [2], Abhiramy Suprasannan [2], Manu Krishna Maniyan Girija [3], Vanyarkina A.S. [2], Moskaleva E.V. [2], Kazantseva E.D. [2], Petrova A.G. [2], Rychkova L.V. [2]

[1] Irkutsk State Medical University (Krasnogo Vosstaniya str. 1, Irkutsk 664003, Russian Federation)
[2] Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)
[3] University of Barcelona (Gran Via de les Corts Catalanes 585, Barcelona, Spain)

Corresponding author:
Evgenia A. Novikova,
e-mail: europe411@mail.ru

ABSTRACT

Background. *Vaccination coverage of children in India is not sufficient since the COVID-19 pandemic (less than 90 %). This may lead to low adherence of parents to children's vaccination.*
The aim. *To study parental attitudes and awareness towards children vaccination programs in India at the COVID-19 pandemic background.*
Methods. *Two hundred and fourteen participants from Kerala state (India) took part in the descriptive cross-sectional study via survey method. The survey was prepared with Google form according the principles of anonymity.*
Results. *Indian parents demonstrated good adherence towards children's vaccination, 98.6 % (95% confidence interval (CI): 95.9–99.5) of them vaccinated their child, and if vaccination appointment had to be rescheduled 84.6 % (95% CI: 79.1–88.8) of them vaccinated children after. Most of Indians (68.7 %; 95% CI: 62.1–74.5) preferred to vaccinate children in state clinics, however, 28.5 % (95% CI: 22.8–34.8) chose private clinics. Information about diseases that vaccines can prevent, vaccine safety, and side effects 47.2 % (95% CI: 40.6–53.8) of parents got from public pediatricians, 50.9 % (95% CI: 44.2–57.5) – from private pediatricians, and 10.3 % (95% CI: 6.8–15.0) – from complementary and alternative medicine practitioners. Over 80 % of Indians were informed about vaccination through mass media (83.6%; 95% CI: 78.1–87.9). Indian parents showed low awareness about vaccination, because 63.1 % (95% CI: 56.4–69.2) of parents wanted to know more about vaccination. Moreover, before vaccination 21.5 % (95% CI: 16.5–27.4) of them were not informed by a doctor about health benefits and possible risks for their children.*
Conclusion. *In the COVID-19 pandemic Indian parents showed good attitude towards vaccination and low awareness in vaccination questions.*

Key words: *vaccination, vaccine prevention, children, parents, vaccine attitude, vaccine awareness, COVID-19*

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ОТНОШЕНИЕ И ОСВЕДОМЛЁННОСТЬ ИНДИЙСКИХ РОДИТЕЛЕЙ ИЗ ШТАТА КЕРАЛА О ВАКЦИНАЦИИ ДЕТЕЙ В УСЛОВИЯХ ПАНДЕМИИ COVID-19

Ааромал Сурешкумар [1], Новикова Е.А. [2], Абхирами Супрасаннан [2], Ману Кришна [3], Ваняркина А.С. [2], Москалева Е.В. [2], Казанцева Е.Д. [2], Петрова А.Г. [2], Рычкова Л.В. [2]

[1] ФГБОУ ВО «Иркутский государственный медицинский университет» (664003, г. Иркутск, ул. Красного Восстания, 1, Россия)

[2] ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, Иркутск, ул. Тимирязева, 16, Россия)

[3] Университет Барселоны (г. Барселона, ул. Gran Via de les Corts Catalanes, 585, Испания)

Автор, ответственный за переписку: **Новикова Евгения Анатольевна,** e-mail: europe411@mail.ru

РЕЗЮМЕ

Введение. *С начала пандемии COVID-19 в Индии наблюдается снижение охвата вакцинацией детей (менее 90 %), что может привести к снижению приверженности родителей вакцинации.*

Цель. *Оценить отношение родителей из Индии к вакцинации детей в условиях пандемии COVID-19 и их осведомлённость в вопросах вакцинации.*

Методы. *В описательном поперечном исследовании методом опроса с помощью Google Форм приняли участие 214 родителей из штата Керала, Индия.*

Результаты. *Родители из Индии продемонстрировали хорошую приверженность вакцинации, поскольку 98,6 % родителей (95%-й доверительный интервал (95% ДИ): 95,9–99,5) вакцинируют своего ребёнка, а в случаях нарушения графика иммунопрофилактики 84,6 % (95% ДИ: 79,1–88,8) родителей стараются наверстать пропущенную прививку как можно скорее. Большинство индийцев – 68,7 % (95% ДИ: 62,1–74,5) – проводят вакцинацию ребёнка в государственных клиниках, однако 28,5 % (95% ДИ: 22,8–34,8) родителей предпочитают частные медицинские учреждения. Информацию о вакциноуправляемых инфекциях, безопасности вакцин и побочных эффектах большая часть родителей получает от специалистов здравоохранения: 47,2 % (95% ДИ: 40,6–53,8) – от педиатров из государственных учреждений здравоохранения; 50,9 % (95% ДИ: 44,2–57,5) – от педиатров из частных клиник; 10,3 % (95% ДИ: 6,8–15,0) – от врачей альтернативной медицины. Примечательно, что более 80 % индийцев информированы о пользе вакцинопрофилактики через средства массовой информации (83,6 %; 95% ДИ: 78,1–87,9). На фоне хорошего отношения к иммунопрофилактике индийские родители хотят знать больше о вакцинации (63,1 %; 95% ДИ: 56,4–69,2). На недостаточную осведомлённость в вопросах вакцинации также указывает то, что перед вакцинацией ребёнка 21,5 % (95% ДИ: 16,5–27,4) из них не были проинформированы врачом о пользе вакцины для здоровья и возможных рисках.*

Выводы. *В условиях пандемии COVID-19 родители из Индии (штат Керала) продемонстрировали позитивное отношение к вакцинации детей и недостаточную осведомлённость в вопросах вакцинации.*

Ключевые слова: *вакцинация, вакцинопрофилактика, дети, родители, отношение к вакцинации, приверженность вакцинации, COVID-19*

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INTRODUCTION

Vaccines help protecting children from vaccine-preventable diseases and improve child survival and reduce morbidity. To this day the coverage for many essential vaccines is higher than 80 % [1]. The triple vaccine against diphtheria, tetanus and pertussis (DTP3) is used as the key metric for global vaccination coverage, because it is a good indicator for access to routine immunization services [1]. Global target coverage of children according to the World Health Organization (WHO) should be not less than 90 % with three doses of DTP3 [2]. In conformity with WHO the health Mission Indradhanush of Indian Government has an aim to increase full immunization coverage to 90 % through focus on unvaccinated and partially vaccinated children in pockets of low immunization coverage in high risk and hard-to-reach areas [3].

The rates of vaccination of children around the world and in India inter alia are not sufficient. According to recent reports in 2022 the global vaccination coverage was 84 %, in 2021 – 81 %, in 2020 – 83 % [4], and such low coverage may be explained by impacts of the COVID-19 pandemic. India has not achieved 90 % coverage during the COVID-19 pandemic showing 85 % in 2020 and 2021, in comparison with 91 % in 2019 [1]. Interestingly, at the same time attitude to vaccination remains positive, because 92 % people in the world, and 98 % of Indians think that it is important for children to be vaccinated [1]. Besides 98 % Indian parents think vaccines are safe, and 78.9 % trust healthcare specialists (Fig. 1).

The Universal Immunization Program in India is provided free of cost by Ministry of Health and Family Welfare. National Immunization Schedule includes 11 vaccines against diphtheria; pertussis; tetanus; polio; measles; tuberculosis; hepatitis; meningitis and pneumonia caused by Haemophilus influenza type B; rubella and rotavirus diarrhea in selected states and Japanese encephalitis in endemic districts [3].

India does not have mandatory vaccination on a national basis; however, vaccination policies differ across states [5]. Even though vaccination policies in India is recommended, not mandatory, because of rumors about health risks on social media, in 2018 Kerala state required schools to submit students' annual vaccination records as a response to a poor turnout for a new measles-rubella vaccine [6]. India puts efforts for creating awareness and community engagement via engagement with key media houses, advocacy with important opinion makers including religious leaders and local influencers, development of a pictorial National Immunization Schedule [3].

This work is a part of the survey of the Laboratory of Infectiology and Immunoprophylaxis in Pediatrics (The Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk, Russia) about attitudes towards children's vaccination of parents from Russia (Irkutsk region) and India (Kerala state) [7–10]. Since we already made in-depth study that involved Rus-

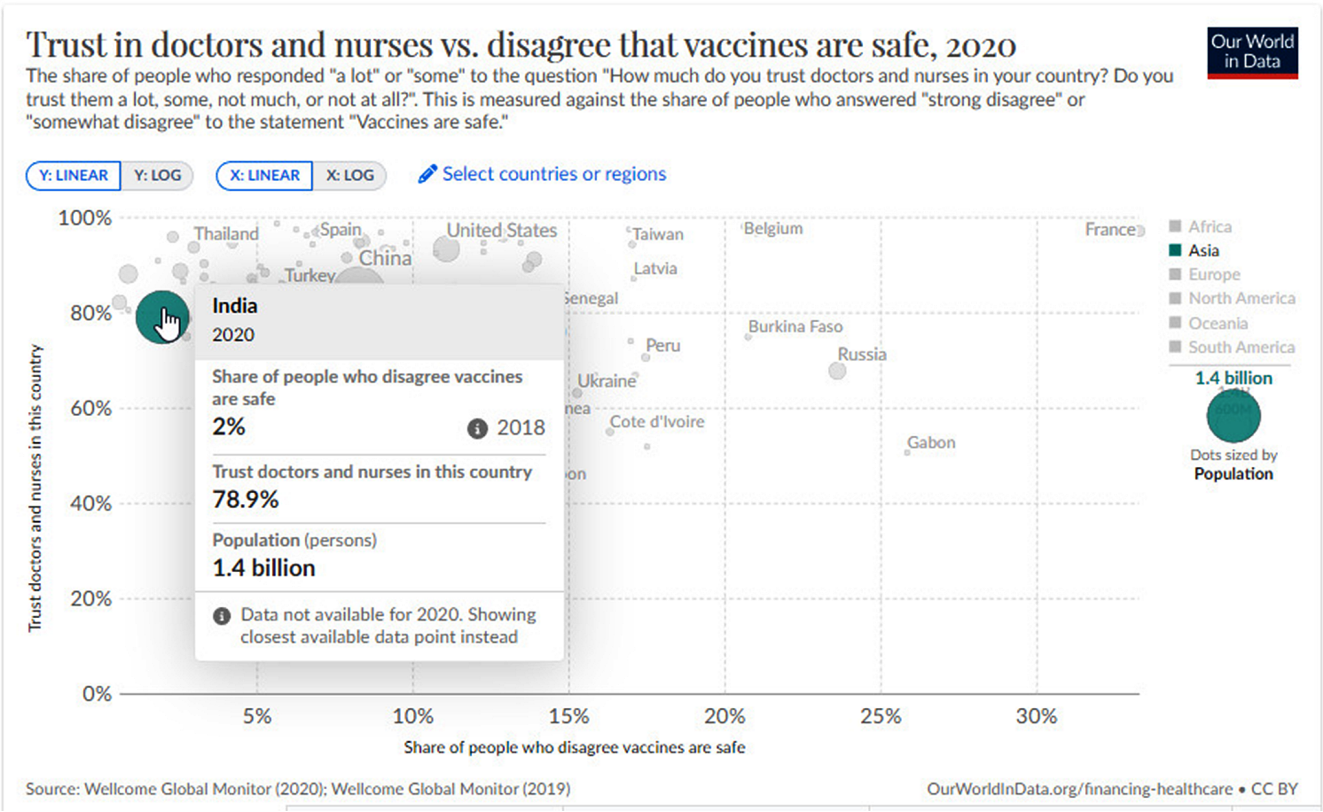

FIG. 1.

The opinion of people in India about vaccine safety and their trust in healthcare specialists (The Welcome Global Monitor, 2020) [1]

sian parents [7] a purpose of this work was to describe in detail the attitude and awareness of parents from India towards children's vaccination on the background of COVID-19 pandemic.

METHODS

Two hundred and fourteen participants from India (Kerala state) took part in the descriptive cross-sectional study. The survey was prepared with Google form according to the principles of anonymity. Data were collected during the COVID-19 pandemic from April 1 to July 6, 2020. The questionnaire contained 17 questions with multiple choice options including age (< 20 y.o., 21–30 y.o., 31–40 y.o., > 40 y.o.) and gender of participants, their educational stage (school, college, university), financial situation (lower middle class, middle class, upper middle class, wealthy), occupational status of family members (health worker, teacher, kindergarten worker), number of children (one, two, three, four or more); and questions about awareness and attitude towards vaccination. The link was sent to the heads of the universities and via social media. We obtained 214 responses, and a total of 214 participants were included for final statistical analysis, so the effective response rate was 100 %. There were no responses missing data and responses with obviously false answers.

The study protocol, questionnaire and consent form were approved by the ethics committee of the "Scientific Center for Family Health and Human Reproduction Problems". Qualitative data were reported as absolute values and percentages. 95 % confidence interval (95% CI) was calculated using the website for statistical computation "VassarStats" [11].

RESULTS AND DISCUSSION

Sociodemographic characteristics of participants are presented in the Table 1. Most of them were women (68.2 %; 95% CI: 61.7–74) and young adults aged 21 to 40 y. o. (79.9 %; 95% CI: 74.0–84.7), university graduates (61.7 %: 95% CI: 55–67.9), belonging to the middle class 66.8 % (95% CI: 60.2–72.7) with one child (65.9 %; 95% CI: 59.3–71.9). Family members of respondents had high education, were health workers (44.9 %; 95% CI: 38.3–51.5), teachers (57.9 %; 95% CI: 51.2–64.3) or kindergarten workers (30.4 %; 95% CI: 0.16–0.24).

Attitude and awareness of Indian parents towards vaccination is shown in the Table 2. Parents' attitude towards vaccinations during the COVID-19 pandemic was positive. Almost all Indians in Kerala state supported children's vaccination and vaccinated their children (n = 211; 98.6 %; 95% CI: 95.9–99.5), and only 1.4 % (n = 3; 95% CI: 0.48–4.04) of parents did not. This data exceeds official numbers of vaccination coverage date cross the country in 2020 (85 %) [1].

Among those parents who vaccinated children (n = 211) 92.5 % (95% CI: 88.2–95.3) followed the Indian Immunization Schedule and knew about the time of vaccinations, but 6.1 % (95% CI: 3.5–10.1) preferred not to care about the schedule. If vaccination appointment had to be rescheduled 84.6 % (95% CI: 79.1–88.8) of Indians vaccinated children later, and just 5.6 % (95% CI: 3.2–9.5) did not want to overload the child's immune system and cancelled vaccine intake.

The sources of information about vaccination for Indians were numerous. Pediatricians provided parents with all necessary information (98.1 %; 95% CI: 95.2–99.2). Among these 47.2 % pediatricians worked in the public health system (95% CI: 40.6–53.8), and 50.9 % (95% CI: 44.2–57.5) – in private clinics. According to The Welcome Global Monitor, Indians consider healthcare specialists as a reliable source of information (Fig. 1), and our results confirm this. Besides, it is worth noting that 10.3 % (95% CI: 6.8–15.0) of parents got informed about vaccination from homeopaths as complementary and alternative medicine practitioners. Homeopathy is included in the six Indian systems of medicine prevalent and practiced in India that is called AYUSH (Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy) [12]. Positive attitude towards vaccination from the alternative medicine specialists whom people trust besides healthcare professionals provides adherence to national immunization in India.

Along with this most of Indians got informed about vaccinations through mass-media (83.6 %; 95% CI: 78.1–87.9), especially Internet/online social media (52.8 %; 95% CI: 46.1–59.3). There is a global trend when people are asking questions about vaccines and are looking for answers online [1]. Since India has high immunization coverage our data demonstrates excellent work of mass communication in India during the COVID-19 pandemic.

All vaccines are given free of charge under the National Immunization Schedule, and so most of Indians (68.7 %; 95% CI: 62.1–74.5) preferred to vaccinate children in state clinics without a fee, however, some of them chose private-pay clinics 28.5 % (95% CI: 22.8–34.8). Private clinics provide around 21 % of vaccinations in urban centers of India and are important partners in achieving high vaccination coverage [13]. Meanwhile private physicians may contribute to the low vaccination coverage, because they do not strictly follow vaccination schedules if there are concerns about parents' ability to pay (45 % of physicians), and do not administer more than two injections in the same visit (60 %) [13].

Before vaccination 65.4 % (95% CI: 58.8–71.4) of parents had been warned by a doctor about diseases that can be prevented by vaccines and risks of vaccinating kids; 13.1 % (95% CI: 9.2–18.2) of parents were informed only about diseases that vaccines can prevent; 21.5 % (95% CI: 16.5–27.4) of parents were not informed by a doctor at all. Getting no information from doctors in turn leads to poor parental awareness. Reasonably

TABLE 1

SOCIODEMOGRAPHIC CHARACTERISTICS OF PARTICIPANTS

Variables	*N* = 214	%	95% CI
Gender			
Male	68	31.8	25.9–38.9
Female	146	68.2	61.7–74
Age			
< 20 y. o.	3	1.4	0.5–4.0
21–30 y. o.	85	39.7	33.4–46.4
31–40 y. o.	86	40.2	33.8–46.8
> 40 y. o.	40	18.7	14.0–24.4
Educational stage			
School	17	7.9	5.0–12.3
College	65	30.4	24.6–36.8
University	132	61.7	55–67.9
Financial situation			
Poverty	1	0.5	0.08–2
Lower middle class	7	3.3	1.5–6.6
Middle class	143	66.8	60.2–72.7
Upper middle class	47	22	16.9–27.9
Wealthy	16	7.5	4.6–11.8
Occupational status of family members			
Health workers	96	44.9	38.3–51.5
Teachers	124	57.9	51.2–64.3
Kindergarten workers	65	30.4	0.16–0.24
None of these	37	17.3	24.6–36.8
Number of children			
One	141	65.9	59.3–71.9
Two	56	26.2	20.7–32.4
Three	15	7	4.2–11.2
Four and more	2	0.9	0.2–3.3

Note. y. o. – years old; 95% CI – 95 % confidence interval; N – number of participants.

63.1 % (95% CI: 56.4–69.2) of Indian parents in our survey wanted to know more about vaccination. It may be explained by low awareness of doctors themselves about vaccination health benefits. Multicenter cross-sectional survey revealing awareness and attitude of Indian healthcare providers towards annual influenza vaccination showed that 42.95 % of physicians had low level of awareness about influenza vaccination (*n* = 780) [14]. Physicians did not prescribe influenza vaccines to patients due to fear of side effects (16.54 %), cost (15.64 %), lack of awareness about availability (15.38 %), absence of belief that it is beneficial (14.36 %), history of side effects (13.46 %), and patients' fear of needles (11.28 %) [14]. This indicates the need to expand vaccine awareness campaigns in India and pay attention to educational strategies among physicians. From the experience of Russian colleagues (S.D. Timoshkova et al.), the immunization training course for doctors not only raises their vaccination awareness but improves vaccination coverage [15]. After two years training of pediatri-

TABLE 2

ATTITUDE AND AWARENESS OF INDIAN PARENTS TOWARDS VACCINATION

Variables	N	%	95% CI
Describe your attitude towards vaccinations.			
I follow Immunization Schedule and know about the time of vaccinations	198	92.5	88.2–95.3
I don't follow Immunization Schedule and don't know about the time of vaccinations, our doctor cares about the Immunization Schedule	13	6.1	3.5–10.1
I don't vaccinate my child	3	1.4	0.48–4.04
If you ever rescheduled vaccinations, have you vaccinated children after resolving conditions?			
Yes, I try to vaccinate my child as soon as possible	181	84.6	79.1–88.8
No, I don't want to overload the child's immune system	12	5.6	3.2–9.5
No, I never reschedule vaccinations	21	9.8	6.5–14.5
Where do you go to get vaccinated your child?			
State clinics without a fee	147	68.7	62.1–74.5
Private clinics with a fee for getting vaccinated	61	28.5	22.8–34.8
Other	6	2.8	1.2–5.9
Before vaccination			
A doctor informs about the disease that can be prevented by the vaccine, about vaccine safety, and side effects	140	65.4	58.8–71.4
A doctor informs only about the disease that can be prevented by the vaccine	28	13.1	9.2–18.2
A doctor gives no information	46	21.5	16.5–27.4
Where do you get information about vaccinations?			
From a pediatrician working in a private clinic	109	50.9	44.2–57.5
From a pediatrician working in the public health system	101	47.2	40.6–53.8
From doctors of other specialty	62	29.0	23.3–35.3
From a homeopath	22	10.3	6.8–15.0
From friends/relatives with a medical background	129	60.3	53.6–66.6
From friends/relatives without a medical background	18	8.4	5.3–12.9
From mass-media (television, radio, newspapers, and magazines)	179	83.6	78.1–87.9
From Internet/social network	113	52.8	46.1–59.3
From Flyers and disk	2	0.9	0.25–3.3
Do you want to know more about vaccination?			
Yes	135	63.1	56.4–69.2
No	79	36.9	30.7–43.5

Note. y. o. – years old; 95% CI – 95 % confidence interval; N – number of participants.

cians at the Moscow Outpatient Department immunization coverage of children attached to this department increased for whooping cough and measles by 11 % and for rubella by 4 %.

When analyzing sociodemographic characteristics of participants, their attitude and awareness towards vaccination we have found some studies demonstrating that younger and more educated parents are less positive about vaccination [16], but we did not find any confirmation of that. In our study, most parents (more than 60 %) were young and well educated, however they had good adherence to children vaccination.

CONCLUSION

Indian parents supported children vaccination and had good attitude towards vaccines of the National Immunization Schedule in the COVID-19 pandemic. Mass media along with healthcare specialists promoted adherence to childhood immunization for Indians. However, parents consider their awareness about children's vaccination as poor, which may be explained by insufficient knowledge of doctors in vaccination questions. Increasing vaccination compliance of Indian healthcare specialists will provide better awareness and vaccination coverage in India.

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Conflict of interest

The authors declare no potential conflicts of interest.

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Information about the authors

Ajitha Sureshkumar Aaromal – MD, Resident at the Department of Neurosurgery, Irkutsk State Medical University, e-mail: aaromalas@gmail.com, https://orcid.org/0000-0002-8655-1780

Evgenia A. Novikova – Junior Research Officer at the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: europe411@mail.ru, https://orcid.org/0000-0002-9353-7928

Suprasannan Abhiramy – MD, Volunteer at the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: abhiramyroses@gmail.com, https://orcid.org/0000-0001-5343-3019

Maniyan Girija Manu Krishna – MD, Student of the Master Global Health, University of Barcelona, e-mail: manumedic27@gmail.com, https://orcid.org/0000-0002-4843-6538

Anastasiya S. Vanyarkina – Cand. Sc. (Med.), Research Officer at the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: nasty-191@yandex.ru, https://orcid.org/0000-0001-8434-1600

Ekaterina V. Moskaleva – Cand. Sc. (Med.), Research Officer at the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: mkatena@mail.ru, https://orcid.org/0000-0003-4196-0713

Ekaterina D. Kazantseva – Clinical Research Assistant at the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: kat.smile7@yandex.ru, https://orcid.org/0000-0003-0692-2295

Alla G. Petrova – Dr. Sc. (Med.), Professor, Head of the Laboratory of Infectology and Immunoprophylaxis, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: rudial75@gmail.com, https://orcid.org/0000-0002-7965-8061

Lyubov V. Rychkova – Dr. Sc. (Med.), Professor of the RAS, Corresponding Member of the RAS, Director, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-0117-2563

PREVALENCE OF SLEEP DISORDERS IN TEENAGE GIRLS IN IRKUTSK (QUESTIONNAIRE DATA)

Bolshakova S.E.,
Madaeva I.M.,
Berdina O.N.,
Khramova E.E.,
Bugun O.V.,
Rychkova L.V.

Scientific Centre of Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)

Corresponding author:
Svetlana E. Bolshakova,
e-mail: sebol@bk.ru

ABSTRACT

Background. *Adequate sleep ensures a person's physical and psycho-emotional well-being. Adolescence is one of the critical stages of life. The exclusive attention of specialists and leveling the impact of adverse factors on the body during this period is the key to the proper development and preservation of the health of adolescents. Meanwhile, sleep problems in teenage girls remain poorly understood.*
The aim. *To study the features of the sleep regime and quality of sleep of teenage girls in the city of Irkutsk.*
Materials and methods. *A survey of 422 teenage girls in the city of Irkutsk was conducted using a translated version of a questionnaire about adolescent sleep habits to subjectively assess their sleep and wakefulness. Two groups were formed: group I – girls with sleep problems (n = 171); group II – girls without sleep problems (n = 251).*
Results. *Sleep disorders among teenage girls in the city of Irkutsk occurred with a frequency of 40.52 %. In most cases, a complex effect of various unfavorable factors on the sleep process has been identified. The features of sleep hygiene of teenage girls are reflected. The adolescents with sleep disorders we examined were characterized by higher rates of sleep latency, later bedtime, earlier awakening, decreased time of night sleep, as well as changes in the sleep shift indicator towards its increase.*
Conclusions. *The conducted survey allows us to draw a conclusion that the issues of sleep schedule and quality of female adolescents in Irkutsk are relevant and should undergo a more detailed comprehensive study. Considering the potential risks to health formation, including reproductive health in female adolescents, more attention should be devoted to proactively identifying sleep-related disorders in adolescents and providing timely interventions to address them.*

Key words: *female adolescents, sleep disorders, impaired sleep-wakefulness, reproductive health*

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ОСОБЕННОСТИ РЕЖИМА И КАЧЕСТВА СНА ДЕВОЧЕК-ПОДРОСТКОВ ГОРОДА ИРКУТСКА

Большакова С.Е.,
Мадаева И.М.,
Бердина О.Н.,
Храмова Е.Е.,
Бугун О.В.,
Рычкова Л.В.

ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)

Автор, ответственный за переписку:
Большакова Светлана Евгеньевна,
e-mail: sebol@bk.ru

РЕЗЮМЕ

Обоснование. *Полноценный сон обеспечивает физическое и психоэмоциональное благополучие человека. Подростковый возраст является одним из критических этапов жизни. Исключительное внимание специалистов и нивелирование воздействия неблагоприятных факторов на организм в этот период является залогом правильного развития и сохранения здоровья подростков. Тем временем проблемы со сном у девочек-подростков остаются малоизученными.*

Цель исследования. *Изучить особенности режима и качества сна девочек-подростков города Иркутска.*

Материалы и методы. *Проведено анкетирование 422 девочек-подростков города Иркутска с использованием переводной версии опросника о привычках сна подростков для субъективной оценки своего сна и бодрствования. Сформированы две группы: I группа – девочки, имеющие проблемы со сном (n = 171); II группа – девочки, не имеющие проблем со сном (n = 251).*

Результаты. *О проблемах со сном сообщили 40,52 % опрошенных. Выявлено комплексное воздействие различных неблагоприятных факторов на качество сна. Отражены особенности гигиены сна девочек-подростков. Для группы девочек, имеющих проблемы со сном, было характерно повышение показателей латентности сна, более позднее время отхождения ко сну, более раннее пробуждение, сокращение времени сна, а также увеличение сдвига сна.*

Заключение. *Проведённый опрос позволяет сделать вывод о том, что вопросы режима и качества сна девочек-подростков города Иркутска актуальны и должны быть подвергнуты более детальному комплексному изучению. Учитывая потенциальную опасность для формирования здоровья, в том числе репродуктивной функции, девочек-подростков, следует уделять больше внимания активному выявлению у них проблем, связанных со сном, и своевременно проводить мероприятия по их устранению.*

Ключевые слова: *девочки-подростки, расстройства сна, нарушение цикла «сон-бодрствование», репродуктивное здоровье*

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INTRODUCTION

Sleep is one of the basic components of any person's health. No one can overestimate the role of sleep in ensuring the vital activity of the human body at any age [1, 2]. It is of particular importance in childhood and adolescence, a period of intense organism formation, physical and psycho-emotional maturation [3, 4]. Currently, sleep disorders in adolescents are quite widespread, occurring in about half of those examined and tending to increase, which cannot but cause concern about their further development and preservation of health, as the adverse consequences can be very serious [5–7]. A decrease in the quality of sleep is associated with the formation of many pathological processes. In particular, it has been proven that sleep disorders can cause cognitive impairment, social maladjustment, acute and exacerbation of chronic diseases [8, 9]. Multiple studies have shown that at any age period, the incidence of various sleep disorders is higher among females compared to males, which is associated with the activity and gender differences in the functioning of the hypothalamic-pituitary-gonadal system at different periods of life and its subordination to circadian rhythms [6, 10]. That is why active identification of sleep-related disorders among adolescent females is extremely important and necessary for timely implementation of a set of measures to prevent the development of unfavourable consequences for the forming female organism.

THE AIM OF THE STUDY

To study the features of sleep schedule and quality of female adolescents living in the city of Irkutsk.

MATERIALS AND METHODS

A questionnaire survey was conducted among 422 female adolescents 15–17 years old, students in grades 9–11 of 16 schools in Irkutsk, between January and March 2023.

Inclusion criteria: female sex; age 15–17 years; voluntary informed consent to participate in the study; residence in the city of Irkutsk. Exclusion criteria: male sex; age less than 15 years and more than 17 years; refusal to participate in the study.

We used the translated Russian version of the Adolescent Sleep Habits Survey [11] to assess sleep quality. The questionnaire was developed and validated by A.R. Wolfson et al. [12] specifically to study sleep hygiene in adolescents aged 12 to 18 years. It contains questions related to self-assessment of sleep hygiene during the last month, to which one or more answers can be submitted. The questionnaire was to be filled out in a calm environment, at home, at a time convenient for the child, without a time limit. In the survey, the girls made value judgements about their sleep and wakefulness during weekends and weekdays over the past month.

According to the results of the questionnaire, all female adolescents were divided into 2 groups. Group I – girls with sleep disorders ($n = 171$); Group II – girls without sleep disorders ($n = 251$).

The study was conducted in accordance with the provisions of the World Medical Association Declaration of Helsinki (1964, revision 2013) and approved by the Biomedical Ethics Committee of "Scientific Centre for Family Health and Human Reproduction Problems" (Minutes No. 2 dated 08.06.2022). All girls signed informed voluntary consent to participate in the study.

Statistical data processing was performed using Excel spreadsheets (Microsoft Corp., USA) and Statistica application software package, version 6.1 (StatSoft Inc., USA) (license holder – "Scientific Centre for Family Health and Human Reproduction Problems"). The type in the distribution of a characteristic was determined using the Shapiro – Wilk, Lilliefors and Kolmogorov – Smirnov criteria. For variables, median (*Me*) and 25th and 75th quartiles were calculated. Statistically significant differences between two unrelated groups on variables were determined using the parametric Student's t-criterion in case of normal distribution of a characteristic and the non-parametric Mann – Whitney U-criterion – in case of distribution of a characteristic other than normal. Differences between unrelated groups by attributes were determined using the χ^2 criterion with Yeats' continuity correction and Fisher's exact test when the number of at least one of the groups was less than 5. All differences were considered statistically significant at the $p < 0.05$ level.

RESULTS

Sleep disorders among the respondents were found in 171 female adolescents, which was 40.52 %. Among them, 16.96 % girls reported having these disorders for less than 1 month, 33.33 % – for 1 to 6 months, 19.88 % – for 6 to 12 months, 20.47 % – for 1 to 5 years, and 9.36 % – for more than 5 years. However, 36.84 % of the respondents reported worsening sleep disorders in the last 2 weeks, 31.58 % had no worsening and 31.58 % found it difficult to answer.

More than one cause of sleep disorders was reported simultaneously by 73.10 % of adolescents, and only 26.90 % gave one definite cause ($\chi^2 = 44.62$; $p < 0.0001$). Stressful situations (77.78 %), lack of sleep and wakefulness (67.84 %), problems in relationships with classmates (46.78 %) and parents (19.88 %), poor eating habits (31.58 %), health problems (15.20 %) were the most frequently observed by girls.

In the vast majority of cases, girls from both study groups slept alone in the room (77.78 % – in Group I, 84.46 % – in Group II). A tendency was revealed among the group of those with sleep disorders for girls to sleep with a family member, 22.22 and 15.54 %, respectively ($\chi^2 = 3.05$; $p = 0.08$).

In Group I, 77.78 % of the adolescents slept in the same bed every night, almost every night – 18.13 %, quite a few nights – 3.51 %, constantly in different beds – 0.58 %.

In Group II, these indices were 84.46 %, 11.16 %, 3.59 %, and 0.79 %, respectively. Consequently, a tendency towards a higher incidence of sleeping in the same bed among girls without sleep disorders was revealed.

In both study groups, the main reason for going to bed on weekdays was identified by girls as "I want to sleep" (52.05 % – in Group I, 68.13 % – in Group II; $p = 0.0009$), with the second most frequent reason being "finishing my homework" (28.07 and 18.73 %, respectively; $p = 0.02$), in the third place – "finishing social networking" (16.96 and 7.57 %, respectively; $p = 0.003$). Therefore, we can assume that girls with sleep disorders are more likely to perform mental activities immediately before going to bed, which negatively affects the sleep process.

On weekends, the desire to sleep was also the main reason (57.89 % – in Group I, 74.50 % – in Group II; $p = 0.0003$), followed by "finishing social networking" (18.13 and 13.15 %, respectively; $p = 0.16$) and "finishing homework" (9.94 and 4.78 %, respectively; $p = 0.04$). In addition, 4.60 % of adolescents in Group I reported that they finished watching TV programs immediately before going to bed, while in Group II only 2.79 % of respondents gave that answer ($p = 0.02$).

Assessment of sleep latency indicators revealed a statistically significantly longer process of falling asleep in girls with sleep disorders compared to adolescents without these disorders (Table 1).

Analyses of nocturnal sleep timing during the school week revealed that girls with sleep disorders tended to go to bed later than girls without such disorders ($p < 0.0001$). In addition, their maximum bedtime was also significantly later ($p < 0.0001$). Indicators of morning wake-up time also had statistically significant differences between groups: adolescents from Study Group I had a statistically significant earlier wake-up time ($p = 0.02$). Differences were also found on weekends when school was not required: girls with sleep complaints went to bed later ($p < 0.0001$) and woke up later ($p = 0.02$ – for usual time, $p < 0.0001$ – for the latest time) than those without these complaints. The average and minimum sleep duration indices during the school week in both study groups were below the recommended norms, and in the group of girls with sleep disorders they were statistically significantly lower than in girls with normal sleep ($p < 0.0001$ and $p < 0.0001$, respectively). At weekends, sleep in both groups was expectedly longer and fell within the range of normative values for this age group – 8–10 hours, but its minimum duration tended to decrease in adolescents with sleep disorders ($p = 0.08$), and its maximum duration in this group was statistically significantly higher than in female adolescents without sleep disorders ($p < 0.0001$). Sleep shift indices in the groups also had statistically significant differences in the direction of its increase in those with sleep disorders ($p < 0.0001$) (Table 1).

DISCUSSION

The recent increase in the number of people with sleep disorders, including children and adolescents, requires more attentive attitude of physicians of various specialties, primarily pediatricians, to this problem [13, 14]. Of utmost importance to remember that adolescence is one of the critical stages of human life, when the key processes of maturation and restructuring of all systems of the body, accompanied by various hormonal shifts. Of particular importance is the close relationship between the sleep- wakefulness and the menstrual cycle, including through the hypothalamic-pituitary-gonadal system, the activity of which becomes more pronounced during puberty in adolescents and determines gender differences in the characteristics of sleep during this period of life. This stage is characterised in particular by a wave-like secretion of luteinising hormone, with an increase in both the amplitude and frequency of its secretion pulses during night sleep, leading to an increase in estradiol and progesterone levels in girls in the morning hours. Melatonin secreted during sleep has a direct suppressive effect on the secretion of luteinising hormone, and changes in its concentration in female adolescents can cause both sleep disorders and disturbances in the mechanisms of functioning of the hypothalamic-pituitary-gonadal system [15]. Exceptional attention of specialists and levelling the impact of various unfavourable factors on the organism during this period of life is the key to proper development and preservation of female adolescent health, including reproductive potential [16].

The adolescent period is also critical in the process of shaping a child's sleep. A significant proportion of adolescents nowadays have problems related to sleep disorders, either short-term or over a long period of time [3, 7]. The incidence of sleep disorders that was revealed by this study was quite high and comparable with the rates obtained earlier in similar studies. For instance, according to various sources, sleep disorders among adolescents occur with a frequency ranging from 7 to 40 % [13, 17]. A number of studies have reported higher rates. According to K.A. Gazenkampf et al. (2019), nocturnal sleep disorders were observed in 52 % of high school students [18], and according to F. Brooks et al. (2015), among female adolescents in England, 49 % reported sleep disorders [19]. It should be emphasised that the female adolescents who were surveyed more often reported sleep disorders for quite a long time, namely from 1 to 6 months, which suggests a prolonged influence of unfavourable conditions on the development of the organism. At the same time, there was a tendency for sleep problems to worsen over time, which probably indicates the occurrence of any functional disorders of the organism.

It is known that the important factors influencing the quality of sleep are compliance with the sleep and wakefulness schedule, daily sleep time, room temperature during sleep, lighting and noise level in the room during sleep, the impact of so-called blue radiation (use of social networks just before going to bed, watching TV, studying at the computer), psycho-emotional stress, the level of physical activity, the nature of nutrition, the use of tonic drinks, alcohol, smoking [4, 20]. Having studied the sleep habits of the surveyed girls, it was concluded that in the vast majority of cases there was a complex impact of various unfavourable factors on the sleep process, which often have the possibility

TABLE 1

PECULIARITIES OF FEMALE ADOLESCENT GIRLS' SLEEP PATTERNS AT WEEKDAYS AND WEEKENDS

Indicators	Group I (*n* = 171)	Group II (*n* = 251)	*p*
Sleep latency during the school week (min)			
Mean	30.00 (15.00; 60.00)	15.00 (10.00; 30.00)	0.0001*
Minimum	10.00 (5.00; 30.00)	10.00 (5.00; 15.00)	0.0001*
Maximum	60.00 (30.00; 120.00)	40.00 (20.00; 60.00)	0.0001*
Weekend sleep latency (min)			
Mean	30.00 (12.00; 60.00)	15.00 (10.00; 30.00)	0.0001*
Minimum	10.00 (5.00; 30.00)	10.00 (5.00; 15.00)	0.0004*
Maximum	60.00 (30.00; 120.00)	60.00 (20.00; 60.00)	0.0001*
Bedtime during the school week			
Regular	23:40 (23:00; 00:30)	23:00 (22:30; 24:00)	0.0001*
Earliest	22:00 (21:00; 23:00)	22:00 (21:05; 22:40)	0.81
At the latest	02:00 (01:00; 03:30)	01:00 (23:40; 02:00)	0.0001*
Wake-up time during the school week			
Regular	06:30 (06:00; 07:00)	06:30 (06:05; 07:00)	0.93
Earliest	06:00 (05:30; 06:30)	06:00 (05:40; 06:30)	0.02*
At the latest	07:00 (06:40; 07:30)	07:00 (06:40; 07:30)	0.45
Bedtime on weekends			
Regular	00:00 (23:00; 01:30)	00:00 (23:00; 01:00)	0.18
Earliest	22:30 (22:00; 23:00)	22:30 (22:00; 23:00)	0.76
At the latest	03:00 (01:00; 04:00)	02:00 (00:00; 03:00)	0.0001*
Wake-up time on weekends			
Regular	10:00 (09:10; 11:30)	10:00 (09:00; 11:00)	0.02*
Earliest	08:40 (07:30; 09:30)	08:30 (08:00; 09:00)	0.93
At the latest	12:30 (11:30; 14:00)	12:00 (10:30; 13:00)	0.0001*
Length of night sleep during the school week (h)			
Mean	6.00 (5.50; 7.00)	7.00 (6.20; 8.00)	0.0001*
Minimum	4.00 (3.00; 5.50)	6.00 (5.00; 7.00)	0.0001*
Maximum	8.00 (7.50; 10.00)	8.40 (8.00; 9.00)	0.86
Night sleep duration on weekends (h)			
Mean	9.00 (8.00; 10.00)	9.00 (8.00; 10.00)	0.62
Minimum	8.00 (6.00; 8.00)	8.00 (7.00; 9.00)	0.08*
Maximum	12.00 (10.00; 12.00)	10.00 (10.00; 12.00)	0.0001*
Sleep shift, min	150.00 (90.00; 240.00)	120.00(60.00; 180.00)	0.0001*

Note. Bedtime and wake-up time are presented in 24-hour format; data are presented as Me (25th; 75th quartiles);* – statistically significant differences between groups, $p < 0.05$.

of levelling. Of particular note, this study has demonstrated increased social media communication using a smartphone by girls with sleep disorders just before bedtime, contributing to increased sleep latency and decreased sleep duration. To avoid negative effects to the sleep quality, it is recommended to avoid any use of screens for 1 hour before going to bed. The measures undertaken to correct sleep hygiene, in our opinion, can reduce the prevalence of sleep disorders in female adolescents, thereby reducing the risk of adverse effects on the organism. Our results are consistent with findings in other studies. Specifically, O.P. Gritsina et al. (2019) in a recent study have revealed that the majority of students do not comply with the sleep regime and hygiene, which affects its qualitative characteristics [2].

Modern living conditions and the development of new technologies make adjustments to the organisation of the daily routine and lifestyle of both adults and children, often leading to a shortening of the daily sleep time. In addition to schoolwork, teens are loaded with extracurricular activities and sports. Besides, nowadays there is an increase in the duration of children's stay at the computer, watching TV, and doing school homework [2, 18, 20]. The increased mental activity just before bedtime in girls with sleep disorders revealed in this study may be one of the factors triggering the development of sleep-related disorders, and may also lead to increased daytime sleepiness, emotional and behavioural disturbances, increased fatigue, reduced attention span and other consequences.

The average duration of sleep required for adolescence has been defined to be between 8 and 10 hours [21]. Furthermore, longer sleep duration is known to occur on weekends than on weekdays as a result of the need for early awakening associated with the start of school activities [22]. Meanwhile, sleep in adolescence is characterised by a number of features such as delayed sleep onset, longer sleep latency and longer wakefulness duration. A later secretion of melatonin and slower accumulation of the homeostatic urge to sleep are associated with these effects [4]. Accordingly, adolescents develop an "evening" chronotype with a tendency to go to sleep later and wake up later [23]. One should consider that the fascination in the late evening hours with various gadgets that produce blue radiation has an even more negative effect on the process of falling asleep and leads to a shorter night's sleep. This study also confirmed a decrease in female adolescent sleep duration scores, but these changes were most pronounced in the group with sleep disorders. These girls had higher sleep latency scores and later bedtimes during both the school week and weekends, resulting in shorter sleep durations. Additionally, as a result of a later wake-up time, the sleep shift values in this group of respondents were higher, reflecting the uneven distribution of sleep time during the week. These findings indicate pronounced impaired sleep-wakefulness in female adolescents with sleep disorders, which increases the risk of adverse effects. The results of this study are consistent with the studies of other authors [2, 13]. In particular, it was previously revealed that about 60–70 % of adolescents sleep less than 8 hours on weekdays, and according to the work of K.A. Gazenkampf et al. (2017), this indicator reached the level of 87 % [18]. Reduced sleep duration in adolescents is also reflected in the work of S.N. Kolomeichuk and L.I. Teplova (2019), where adolescents living in Karelia were surveyed. Gender peculiarities were also revealed in the form of more unfavourable qualitative and quantitative characteristics of sleep in girls compared to boys [7].

CONCLUSION

Notwithstanding all the advances made in recent decades in the study of sleep-related issues, sleep disorders are increasingly common among adolescents and especially among female adolescents. The conducted survey allows us to conclude that the issues of sleep schedule and sleep quality of female adolescents in Irkutsk are important and should be subjected to a more detailed comprehensive study. Considering the potential danger to the health of the growing adolescent organism, in particular the negative impact on the formation of female adolescent reproductive function, more attention should be paid to the active identification of sleep-related disorders in adolescent females and to the timely implementation of a set of measures to address them. As we believe, normalising sleep duration, improving sleep quality and improving sleep conditions can have a significant positive impact on the health of female adolescents.

Limitations of our study may include the small sample size as well as the subjective assessment of sleep of the female adolescents surveyed. We therefore believe that it is necessary to continue studying this problem and additionally apply methods of objective study in female adolescent sleep.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Svetlana E. Bolshakova – Cand. Sc. (Med.), Research Officer at the Laboratory of Somnology and Neurophysiology, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: sebol@bk.ru, https://orcid.org/0000-0002-3104-4212

Irina M. Madaeva – Dr. Sc. (Med.), Chief Research Officer at the Laboratory of Somnology and Neurophysiology, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: nightchild@mail.ru, https://orcid.org/0000-0003-3423-7260

Olga N. Berdina – Cand. Sc. (Med.), Leading Research Officer at the Laboratory of Somnology and Neurophysiology, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: goodnight_84@mail.ru, https://orcid.org/0000-0003-0930-6543

Elena E. Khramova – Cand. Sc. (Med.), Chief External Expert on Pediatric and Adolescent Gynecology of the Ministry of Health of the Irkutsk region, Head of the Department of Adolescent Gynecology of the Clinic, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: aelita-82@mail.ru, https://orcid.org/0000-0002-8042-6276

Olga V. Bugun – Dr. Sc. (Med.), Deputy Director for Clinical Affairs, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-2162-3683

Lyubov V. Rychkova – Dr. Sc. (Med.), Professor of the RAS, Corresponding Member of the RAS, Director, Scientific Centre of Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-0117-2563

ASSESSMENT OF THE ACTUAL NUTRITION OF RURAL ADOLESCENTS OF THE IRKUTSK REGION BECAUSE OF REVISION OF THE NORMS OF PHYSIOLOGICAL NEEDS FOR ENERGY AND NUTRIENTS

Rychkova L.V.,
Pogodina A.V.,
Astakhova T.A.,
Lebedeva L.N.

Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)

Corresponding author:
Tatyana A. Astakhova,
e-mail: tatjana_astahova@mail.ru

ABSTRACT

Background. *The rational nutrition of the child population is given great medical importance as a factor in preserving the health and development of the child. A complete and balanced diet in terms of the content of basic nutrients ensures the normal growth and development of the child's body.*

The aim of the study. *To analyze the actual nutrition of adolescents living in rural areas of the Irkutsk region.*

Materials and methods. *The study involved 69 rural adolescents aged 11–17 years (34 boys, 35 girls). The actual nutrition was studied by the method of 24-hour nutrition reproduction. The energy value of the diet was determined, the nature of the provision of the diet with basic macro- and microelements was studied. The obtained values were compared with the norms of physiological needs for energy and nutrients in 2008 and 2021.*

Results. *The analysis of actual nutrition revealed deviations from the principles of healthy nutrition: insufficient energy value of the diet, deficiency of proteins and fats. The diet of adolescents was characterized by an insufficient content of the main groups of macro- and micronutrients – vitamins A, C and D, essential trace elements, and a deficiency of dietary fiber. The diet of adolescents was characterized by increased sodium intake. The calculated ratio of proteins, fats, carbohydrates indicated a carbohydrate type of diet.*

Conclusion. *Despite the great attention to the problem of balanced nutrition of adolescents, the question of the impact of nutrition on the health of a teenager, considering the regional factor, remains open. Recommendations for the development of a regional program for the organization of proper nutrition for school-age children are of great practical importance.*

Key words: *rural adolescents, actual nutrition, nutrient intake*

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ОЦЕНКА ФАКТИЧЕСКОГО ПИТАНИЯ СЕЛЬСКИХ ПОДРОСТКОВ ИРКУТСКОЙ ОБЛАСТИ В СВЯЗИ С ПЕРЕСМОТРОМ НОРМ ФИЗИОЛОГИЧЕСКИХ ПОТРЕБНОСТЕЙ В ЭНЕРГИИ И ПИЩЕВЫХ ВЕЩЕСТВАХ

Рычкова Л.В.,
Погодина А.В.,
Астахова Т.А.,
Лебедева Л.Н.

ФГБНУ «Научный центр проблем здоровья семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)

Автор, ответственный за переписку:
Астахова Татьяна Александровна,
e-mail: tatjana_astahova@mail.ru

РЕЗЮМЕ

Обоснование. *Рациональному питанию детского населения придаётся огромное практическое значение как основному фактору укрепления здоровья и полноценного развития подрастающего поколения. Полноценное и сбалансированное по содержанию основных пищевых веществ питание обеспечивает нормальный рост и развитие детского организма.*

Цель исследования. *Анализ фактического питания подростков, проживающих в сельской местности Иркутской области.*

Материалы и методы. *В исследовании приняли участие 69 сельских подростков 11–17 лет (34 мальчика, 35 девочек). Фактическое питание было изучено методом 24-часового воспроизведения питания. Определена энергетическая ценность рациона, изучен характер обеспеченности рациона основными макро- и микроэлементами. Полученные значения сравнивали с нормами физиологических потребностей в энергии и пищевых веществах 2008 и 2021 гг.*

Результаты. *Анализ фактического питания выявил отклонения от принципов здорового питания: недостаточная энергетическая ценность рациона, дефицит белков и жиров. Рацион подростков характеризуется недостаточным содержанием основных групп макро- и микронутриентов – витаминов A, C и D, эссенциальных микроэлементов, дефицитом пищевых волокон. Рацион подростков характеризуется повышенным потреблением натрия. Расчётное соотношение белков, жиров, углеводов свидетельствует об углеводном типе питания.*

Заключение. *Несмотря на большое внимание к проблеме сбалансированного питания подростков, вопрос о влиянии питания на состояние здоровья подростка с учётом регионального фактора остаётся открытым. Большое практическое значение приобретают рекомендации для разработки региональной программы по организации правильного питания детей школьного возраста.*

Ключевые слова: *сельские подростки, фактическое питание, потребление нутриентов*

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INTRODUCTION

Balanced nutrition is one of the main factors in supporting the health of the younger generation. The nutritional structure of the population has changed in the last decade: energy expenditures have sharply decreased, and the consumption of the main macro- and microelements has decreased [1]. Russians began to consume less meat, dairy products, vegetables and fruit, while consuming more bakery products and refined products. This has resulted in an increase in the number of nutrient-dependent diseases [2].

Actual nutrition is assessed in most cases among residents of large industrial centres, while research about the actual nutrition of rural residents is fragmentary. A negative trend of decreasing consumption of essential nutrients – dietary fibres and vitamins – by schoolchildren has been revealed according to the studies of the actual nutrition of urban adolescents in different regions of Russia [3]. Similar changes were also revealed in adolescents living in rural areas. Feeding habits are shaped by the child's immediate environment. Ethnicity, family affluence and parents' knowledge of balanced nutrition are essential in this process.

In assessing actual nutrition, the values of the average daily energy and nutrient intake are used and compared with the Physiological Requirements Standards (PRS) adopted in Russia. PRS, approved in 1951, 1968, 1987, 1991 and 2008, periodically undergo the procedure of revision, which is associated with changes in the structure of morbidity of both adult and child population, changes in the socio-economic structure of society and other factors [4, 5].

The study of regional peculiarities of the children's and adolescents' actual nutrition, based on the place of residence, ethnic characteristics, together with the analysis of morbidity is an urgent task.

THE AIM OF THE STUDY

An assessment of the actual nutritional status of rural adolescents.

METHODS

Study design: a single-stage continuous cross-sectional study.

Inclusion criteria for the study group:

- 11–17 years of age;
- permanent residence of the child in the territory of the settlement since birth;
- informed voluntary consent from parents/legal representatives and adolescents over 15 years of age to participate in the study.

Exclusion criteria for the study group:

- age less than 11 years and older than 17 years;
- Failure to thrive (SDS (standard deviation score) < 2 for age and sex according to the World Health Organisation (WHO) reference tables);
- weight deficit (SDS body mass index (BMI) < 5th percentile).

Procedure situation. The study was conducted in November 2020 on the territory of Bayandai settlement, Irkutsk region. Adolescents who attended school during the survey days participated in the study. Informed voluntary consent for participation in the study and processing of personal data was obtained from the participants' legal representatives (parents or guardians) and from children over 15 years of age.

Duration of the study: from November 1, 2020, to December 1, 2020.

Outcomes of the study: the dietary intake – energy value, chemical composition (proteins, fats, carbohydrates, vitamins, microelements, dietary fibre) was assessed.

Ethical review. The study was approved by the Ethics Committee of the Scientific Centre for Family Health and Human Reproduction Problems (Protocol No. 2 dated February 18, 2020).

Assessment of actual nutrition

Actual nutrition was assessed using the 24-hour nutritional replication method [6]. Adolescent surveys were conducted by physicians. According to the instructions received, the physician filled out food diaries that recorded the meals and foods that the adolescent consumed at the main and additional meals during 2 days (1 school day and 1 weekend). Food portion size was assessed using the Food and Meal Portion Album tool, which was shown to the adolescent during the survey [7].

Results obtained by summing the data from the two-day recordings were then averaged. In order to analyse the obtained information about the energy value, quantitative composition of each dish, the data concerning the chemical composition of Russian food products [8] in the information supplement "My Healthy Diet" [9] were used. The data available on the Internet service "My Healthy Diet" regarding the composition of foods are based on the reference book of I.M. Skurikhin and V.A. Tutelian [8].

The data obtained in the study were verified for plausibility. To determine the plausibility of the provided information about the actual nutrition, thresholds corresponding to one standard deviation of the ratio of energy consumption calculated from the questionnaire to the required energy expenditure in per cent for a given sex and age were calculated using the formula:

$$\pm 1SD = \frac{\sqrt{CV_{rEI}^2}}{d} + CV_{pER}^2 + CV_{mTEE}^2,$$

where CV_{rEI} is the variation coefficient of actual energy intake; CV_{pER} – the variation coefficient of the required energy expenditure for a particular age, sex and corresponding physical activity; CV_{mTEE} – the error variation coefficient of daily biological changes in total energy expenditure measured by the water method [10]. If the percentage ratio of actual energy intake to required energy

expenditure was within one standard deviation, the data were considered plausible.

A total of 75 food diaries were included in the study, which provided information about the adolescent's diet. Sixty-nine diaries with plausible information provided were considered for further study.

Energy value and chemical composition data were assessed with consideration of methodical recommendations 2.3.1.2432-08 and 2.3.1.0253-21 "Physiological requirements standards in energy and food substances for different population groups of the Russian Federation" [4, 5].

Statistical analysis

Data were analysed using the IBM SPSS Statistics 21 statistical software package (IBM Corp., USA). The median (*Me*) and 95 % confidence interval (95 % CI) were calculated to compare daily energy and nutrient intake between the formed groups and the general population. A statistically significant difference was considered if the calculated 95 % CI did not include the population mean.

RESULTS

Study sample characteristics

A total of 69 rural adolescents were involved in the study and they provided complete and plausible information about dietary intake: 50.7 % girls, 49.3 % boys (Table 1). Adolescents were divided into age groups: younger schoolchildren – 11–14 years old; older schoolchildren – 15–17 years old [5].

TABLE 1

CHARACTERISTICS OF THE STUDY PARTICIPANTS

Parameters	Adolescents (*n* = 69)
Male, *n* (%)	34 (49.3)
Female, *n* (%)	25 (50.7)
Age, years	15.1 ± 0.8
Sports activities, *n* (%)	16 (23.2)
PAL, *n* (%)	
low	13 (18.8)
average	39 (56.5)
high	16 (23.2)
Body weight, kg	60.6 ± 9.0
BMI, kg/m^2	21.9 ± 2.8
SDS BMI	0.3 ± 0.9
Obesity, *n* (%)	5 (7.2)
WC, cm	73.9 ± 6.8
SDS WC	0.3 ± 0.8

Note: PAL – physical activity level; WC – waist circumference.

Actual consumption of nutrients and energy

The study results of adolescent actual dietary intake are summarised in Tables 2 and 3.

The results of the performed study showed that the energy value of diets, the content of proteins, fats and carbohydrates do not meet the PRS in all adolescents, except for boys 11–14 years old. In this group, the median values of dietary calories as well as protein and fat were above the physiological requirements.

Median values of vitamin intake were below the recommended standards for both boys and girls: there was a deficiency of vitamin A (in terms of retinol – by 41.5–50.2 %) and vitamin C (by 41.2–68.6 %). Considering the fact that the prevalence of vitamin D deficiency is high among the child population of the Russian Federation (RF) and in order to reduce the risk of developing a number of non-communicable diseases, the value of the physiological requirement in the methodical recommendations of the new revision (2021) [5] for this vitamin was increased from 10 to 15 μg/day; thus, the median values of vitamin D intake in our study are 0.7–2.9 μg/day, which is 4.6–9.3 % of the PRS. Mean vitamin E values were within normal limits only in boys in all age groups.

A number of studies have shown an association between insufficient potassium content in the diet of the child population and an increased risk of cardiovascular pathology in adulthood [11–13]. Therefore, the value of the physiological requirement of potassium was increased from 1500 to 2500 mg/day for adolescents in the age group of 11–14 years [5] and from 2500 to 3200 mg/day for adolescents aged 15–17 years; thus, the diet of the studied adolescents was characterised by a deficiency of this element by 18.6–34.1 %. To optimise the calcium : phosphorus ratio, the physiological phosphorus requirement in the methodical recommendations of the new revision was increased to 900 mg/day for all age and gender groups [5]. Considering these changes, the median intake values of this micronutrient were lower than the PRS in the diet of the studied schoolchildren.

A study of mineral intake revealed significant calcium and iodine deficiency in all age and gender groups. Deviations from PRS are more observed in girls in the older age group. The significant excess of sodium intake in all age groups of adolescents is noteworthy.

Physicians have recently started to pay great attention to dietary fibre as one of the important compo-

TABLE 2

ENERGY VALUE AND CHEMICAL COMPOSITION OF ADOLESCENT BOYS' DIETS

Indicators	Boys aged 11–14 (*n* = 9)				Boys aged 15–17 (*n* = 25)			
	PRS-2021/PRS-2008	Me	95 % CI		PRS-2021/PRS-2008	Me	95 % CI	
Energy value, kcal	2500.0/2500	2615.5	2289.1	2941.8	2900.0/2900.0	2714.4	2608.2	2820.7
Proteins, g	75.0/75.0	79.1	69.6	88.6	87.0/87.0	77.0	68.9	85.2
Fats, g	83.0/83.0	85.5	74.6	96.4	97.0/97.0	91.1	83.5	98.8
Carbohydrates, g	363.0/363.0	324.2	280.5	367.8	421.0/421.0	355.7	330.2	381.2
P : F : C		1 : 1.2 : 4.2				1 : 1.2 : 4.6		
Dietary fiber, g	20.0/20.0	13.0	10.7	15.3	22.0/ 20.0	16.4	14.6	18.3
Vitamin A (estrogen receptor), μg	1000.0/1000.0	433.1	122.4	743.8	1000.0/1000.0	568.1	416.0	720.1
Vitamin B_1, mg	1.3/1.3	0.8	0.6	0.9	1.5/1.5	1.1	0.8	1.5
Vitamin B_2, mg	1.5/1.5	0.9	0.7	1.0	1.8/1.8	1.1	0.9	1.3
Vitamin B_5, mg	3.5/3.5	2.0	1.5	2.4	5.0/5.0	2.6	2.0	3.2
Vitamin B_6, mg	1.7/1.7	1.1	0.8	1.3	2.0/2.0	1.3	1.1	1.5
Vitamin B_9, μg	300.0/300.0	62.8	48.3	77.3	400.0/400.0	123.4	73.1	173.8
Vitamin B_{12}, μg	3.0/3.0	3.1	1.6	4.6	3.0/3.0	3.1	2.4	3.9
Vitamin C, mg	70.0/70.0	35.7	11.5	59.9	90.0/90.0	37.1	25.7	48.6
Vitamin D, μg	15.0/10.0	0.7	0.1	1.3	15.0/10.0	0.7	0.4	1.1
Vitamin E (tocopherol equivalent), mg	12.0/12.0	12.2	8.3	16.2	15.0/15.0	15.1	11.6	18.6
Potassium, mg	2500.0/1500.0	2013.9	1586.5	2441.4	3200.0/2500.0	2342.4	2039.3	2645.4
Calcium, mg	1200.0/1200.0	494.3	363.7	624.8	1200.0/1200.0	612.4	518.9	706.0
Magnesium, mg	300.0/300.0	222.3	182.7	261.9	400.0/400.0	318.5	263.4	373.7
Sodium, mg	1100.0/1100.0	2481.2	1716.4	3245.9	1300.0/1300.0	2588.6	2278.9	2898.3
Phosphorus, mg	900.0/1200.0	856.7	680.5	1032.9	900.0/1200.0	1131.4	1001.4	1261.4
Ferrum, mg	12.0/12.0	17.9	13.3	22.6	15.0/15.0	20.1	16.4	23.7
Iodine, μg	130.0/130.0	25.7	19.0	32.4	150.0/150.0	41.9	28.1	55.6

Note. PRS-2021 and PRS-2008 – physiological requirement standards (tabular data according to "Physiological Requirement Standards for Energy and Nutrients for Different Population Groups of the Russian Federation") as of 2021 and 2008, respectively; Me – median; 95 % CI – 95 % confidence interval for the sample median; P : F : C – protein, fat and carbohydrate ratio.

TABLE 3

ENERGY VALUE AND CHEMICAL COMPOSITION OF DIETS OF ADOLESCENT GIRLS

Indicators	Girls aged 11–14 (*n* = 13)				Girls aged 15–17 (*n* = 22)			
	PRS-2021/PRS-2008	Me	95 % CI		PRS-2021/PRS-2008	Me	95 % CI	
Energy value, kcal	2300.0/2300.0	2275.7	2077.7	2473.8	2500.0/2500.0	2373.6	2230.6	2516.7
Proteins, g	69.0/69.0	68.9	64.0	73.8	75.0/75.0	72.2	66.8	77.7
Fats, g	77.0/77.0	74.6	66.0	83.1	83.0/83.0	79.3	73.2	85.4
Carbohydrates, g	334.0/334.0	311.4	282.1	340.7	363.0	315.0	293.0	337.0
P : F : C		1 : 1.1 : 5.1				1 : 1.2 : 4.9		
Dietary fiber, g	20.0/20.0	13.5	11.1	16.0	22.0/20.0	14.5	12.9	16.2
Vitamin A (estrogen receptor), μg	800.0/800.0	332.1	244.9	419.3	800.0/800.0	401.3	282.4	520.2
Vitamin B_1, mg	1.3/1.3	0.7	0.6	0.8	1.3/1.3	1.3	0.3	2.4
Vitamin B_2, mg	1.5/1.5	0.9	0.7	1.1	1.5/1.5	0.9	0.8	1.0
Vitamin B_5, mg	3.5/3.5	1.9	1.6	2.3	4.0/4.0	4.7	-1.3	10.8
Vitamin B_6, mg	1.6/1.6	2.7	-1.0	6.4	1.6/1.6	1.1	0.9	1.3
Vitamin B_9, μg	300.0/300.0	69.7	47.6	91.8	400.0/400.0	86.8	59.1	114.5
Vitamin B_{12}, μg	3.0/3.0	3.1	1.4	4.7	3.0/3.0	4.7	0.1	9.3
Vitamin C, mg	60.0/60.0	41.2	10.0	72.4	70.0/70.0	36.3	26.2	46.5
Vitamin D, μg	15.0/10.0	1.4	0.7	3.5	15.0/10.0	1.0	0.3	2.3
Vitamin E (tocopherol equivalent), mg	12.0/12.0	10.0	7.7	12.4	15.0/15.0	12.5	9.2	15.8
Potassium, mg	2500.0/1500.0	2037.6	1643.6	2431.6	3200.0/2500.0	2131.7	1849.9	2413.6
Calcium, mg	1200.0/1200.0	625.0	436.8	813.2	1200.0/1200.0	561.5	454.0	669.0
Magnesium, mg	300.0/300.0	267.7	222.6	312.8	400.0/400.0	250.9	208.2	293.7
Sodium, mg	1100.0/1100.0	1799.8	1379.7	2219.9	1300.0/1300.0	2064.7	1746.7	2382.8
Phosphorus, mg	900.0/1200.0	914.7	800.4	1029.0	900.0/1200.0	902.9	799.4	1006.3
Ferrum, mg	15.0/15.0	14.5	12.6	16.4	18.0/18.0	17.8	14.2	21.3
Iodine, μg	130.0/130.0	41.0	15.4	66.7	150.0/150.0	29.0	21.2	36.8

Note. PRS-2021 and PRS-2008 – physiological requirement standards (tabular data according to "Physiological Requirement Standards for Energy and Nutrients for Different Population Groups of the Russian Federation") as of 2021 and 2008, respectively; Me – median; 95 % CI – 95 % confidence interval for the sample median; P : F : C – protein, fat and carbohydrate ratio.

nents of nutrition, which contributes to the normalisation of gastrointestinal tract function, reducing the risk of cardiovascular pathology in adulthood, therefore, in the methodical recommendations of 2021 the PRS of dietary fibre constitutes 22 g/day in the age group of 15–17 years [5]. In our study, the median values of dietary fibre intake range from 13.0–16.4 g/day.

In a balanced diet, the calculated ratio of proteins : fats : carbohydrates (P : F : C) should be 1 : 1 : 4. This ratio in our study indicated the predominance of carbohydrate component in the diet of adolescents, especially in girls of 11–14 years of age.

Analysis of consumption of individual products has shown that protein-rich products (meat, fish, poultry) are consumed by an insignificant number of adolescents. For instance, fish is included in the diet in 8.8 % of the studied adolescents, poultry meat – in 17.5 % (Table 4).

TABLE 4

DAILY DIETARY INTAKE CHARACTERISTICS OF THE STUDIED ADOLESCENTS

Indicators	Adolescents (*n* = 69)
1 portion of vegetable/fruit intake, *n* (%)	42 (60.1)
1 portion of fish intake, *n* (%)	5 (7.2)
1 portion of red lean meat intake, *n* (%)	33 (47.8)
1 portion of dairy products intake, *n* (%)	13 (18.8)
1 portion of poultry meat intake, *n* (%)	15 (21.7)
1 portion of sweets and sweetened drinks intake, *n* (%)	63 (91.3)

The leading positions are occupied by sweets and sweetened beverages – 91.3 %.

DISCUSSION

Many studies indicate the presence of changes in the structure and nature of nutrition of the child population in different regions of Russia [14, 15], which is expressed in inadequate intake of energy, macro- and microelements. The results of this study support this fact. In particular, according to I.I. Saldan et al., among adolescents of Altai Territory there is a decrease in the energy value of the diet together with a low consumption of proteins and fats [16]. There are similar changes characterising the diet of adolescents in the Tomsk, Saratov regions and the Republic of Sakha (Yakutia) [17, 18].

Deficiency of vitamins is one of the reasons for the deterioration of children's health, which is associated with impaired metabolism and reduced physical and mental performance. According to Federal Research Center of Nutrition and Biotechnology, the population of the Russian Federation is increasingly deficient in vitamins and microelements. Deficiency of vitamins B is found in 30–40 %, beta-carotene in more than 40 %, and vitamin C in 70–90 % of studied children and adolescents [19]. The results of this study revealed the highest deficiency in vitamins A, C and D among rural adolescents. Similar findings have been revealed by other studies as well. H. Wang et al. revealed vitamin A and C deficiency in Chinese adolescents – 36.1 and 75.5 %, respectively [20]. According to the NHANES (National Health and Nutrition Examination Survey), 95 % of the adolescents studied were diagnosed with vitamin D deficiency [21].

Low vitamin D sufficiency in the child population is a "global, silent, non-communicable pandemic". The results of Russian and international epidemiological studies convincingly prove that the frequency of low vitamin D concentration is at least 70 % (50–90 %) in both adult and child populations [15, 22]. In particular, according to the data of D. Wahl et al., vitamin D deficiency was found in 23.3 % of adolescents under 18 years of age living in Southeast China [22]. A UK study revealed that 70 % of adolescents aged from 14.7 to 16.6 years were vitamin D deficient [15, 23]. Insufficient intake of fatty fish and seafood is one of the causes of this vitamin deficiency.

Micronutrient deficiency in the diet of a modern adolescent is an objective reality of the present time. The organisation of rational nutrition, oriented to adolescent's individual health characteristics, is the foundation for reducing deficiencies in essential nutrients.

An assessment of the micronutrient intake of the rural adolescents' diet revealed an increased intake of sodium. Among rural adolescents in Primorsky Territory, sodium excess significantly exceeded PRS; 72 % of Omsk adolescents were found to exceed the recommended sodium intake standards; about 30 % of adolescents in the Republic of Belarus prefer to salt their food [24]. The proportion of average daily sodium intake in 12–17 years old Chinese adolescents exceeded the corresponding intake rate by 94.4 % [25]. The standard of salt intake in adolescents recommended by WHO experts should be no more than 5 g/day, which is equivalent to 2 g of Na [5].

It is proved that excessive salt intake with food is one of the leading determinants of the formation of high blood pressure and the risk of cardiovascular diseases in adulthood [26, 27]. A number of studies have evidenced an indirect association of excess salt intake on the development of excess body weight: each additional 1 g of salt increased the volume of fluid drunk and also led to an increase in the volume of portions eat-

en [27]. In a number of countries, salt intake is several times higher than the recommended levels, and the addition of salt to cooked food is part of a family tradition rather than a physiological necessity [28].

Calcium intake of the adolescents studied was below the PRS, which may be associated with inadequate intake of milk and dairy products. Low calcium intake is noted among adolescents in Penza and the Republic of Adygea [29]; among adolescents in the Irkutsk Region, calcium content is two times lower than the PRS for adolescents of primary school age [18].

One of the main natural sources of iodine for humans are products of plant and animal origin – milk, eggs, meat, cereals, vegetables. The Irkutsk Region is an iodine-deficient area, and local products cannot fully serve as a source of sufficient intake of this trace element into the body. According to WHO recommendations, iodised salt is used for mass prophylaxis of iodine deficiency, but despite these measures, a significant deficiency of this essential trace element is being observed everywhere among children and adults.

CONCLUSION

The actual nutrition of school-age children remains currently an urgent topic. The results of the study reveal that in rural conditions the diet of adolescents is characterised by imbalance in the main macro- and microelements. Low energy values and predominance of carbohydrate type of nutrition are observed. Deficiency of the most important vitamins (A, C, D), as well as microelements (calcium, iodine) has been revealed. Excessive sodium intake is particularly alarming. Data on the actual diet of rural adolescents may become an important component for the development of methodological recommendations for improving the nutrition of children and adolescents, followed by health education among the child population.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Lyubov V. Rychkova – Dr. Sc. (Med.), Professor of the RAS, Corresponding Member of the RAS, Director, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: iphr@sbamsr.irk.ru, https://orcid.org/0000-0002-0117-2563

Anna V. Pogodina – Dr. Sc. (Med.), Chief Research Officer, Head of the Laboratory of Pediatrics and Cardiovascular Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: pogodina_av@inbox.ru, https://orcid.org/0000-0001-8533-3119

Tatyana A. Astakhova – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Pediatrics and Cardiovascular Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: tatjana_astahova@mail.ru, https://orcid.org/0000-0003-1427-4734

Lyudmila N. Lebedeva – Clinical Laboratory Assistant at the Laboratory of Pediatrics and Cardiovascular Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: leb_46@mail.ru, https://orcid.org/0000-0002-7289-6024

PREVENTIVE MEDICINE

SEX AND AGE SPECIFICITIES OF THE DYNAMICS OF ANTHROPOMETRIC INDICATORS CHARACTERIZING OBESITY (ACCORDING TO A PROSPECTIVE EPIDEMIOLOGICAL RESEARCH)

Tsygankova D.P.,
Bazdyrev E.D.,
Nakhratova O.V.,
Tsenter I.M.,
Gaziev T.F.,
Indukaeva E.V.,
Artamonova G.V.,
Barbarash O.L.

Research Institute of Complex Problems of Cardiovascular Diseases (Sosnovy blvd 6, Kemerovo 650002, Russian Federation)

Corresponding author:
Iosif M. Tsenter,
e-mail: tsenter1998@mail.ru

ABSTRACT

Background. *Overweight and obesity significantly increase the risk of premature death and the development of chronic diseases. Many anthropometric indices have been developed to verify obesity, although the best among them remains undetermined.*

The aim of the study. *To determine the sex and age specificities of the dynamics of anthropometric indicators characterizing obesity.*

Materials and methods. *The program was implemented in the period from 2015 to 2020. It provided for the implementation of sample research. The baseline research included 1,124 women and 476 men. The average age was 54.9 ± 9.75 years and 52.6 ± 10.0 years, respectively. To identify gender specificities, all participants were divided into three age groups: 35–49 years old, 50–59 years old, and 60–70 years old. The observation period was 3 years.*

To determine the level of visceral fat, the VS-532 fat mass analyzer (Tanita Health Equipment HK Ltd., Hong Kong) was used. Body mass index (BMI), waist-to-hip ratio (WHR), visceral obesity index (VOI) were also calculated. Statistical processing of the results was carried out using the program Statistica 6.0 (StatSoft Inc., USA).

Results. *New cases of obesity developed in 30.6 % of the surveyed. There was an increase in the prevalence of obesity according to the criteria of waist circumference (by 8.9 %) and visceral fat level (by 5.4 %) and a decrease in the number of people who are obese according to WHR – by 4.2 %. Of all the indicators, only VOI showed a statistically significant decrease in the mean values over the observed period, while BMI, waist circumference and visceral fat level showed an increase.*

Conclusion. *It is necessary to apply various criteria for the diagnosis of obesity, since individual indices are not able to fully reflect the gender and age specificities of the distribution of fat in the body.*

Key words: *obesity, anthropometry, epidemiology, diagnostics*

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ПОЛОВОЗРАСТНЫЕ ОСОБЕННОСТИ ДИНАМИКИ АНТРОПОМЕТРИЧЕСКИХ ПОКАЗАТЕЛЕЙ, ХАРАКТЕРИЗУЮЩИХ ОЖИРЕНИЕ (ПО ДАННЫМ ПРОСПЕКТИВНОГО ЭПИДЕМИОЛОГИЧЕСКОГО ИССЛЕДОВАНИЯ)

Цыганкова Д.П.,
Баздырев Е.Д.,
Нахратова О.В.,
Центер И.М.,
Газиев Т.Ф.,
Индукаева Е.В.,
Артамонова Г.В.,
Барбараш О.Л.

ФГБНУ «Научно-исследовательский институт комплексных проблем сердечно-сосудистых заболеваний» (650002, г. Кемерово, Сосновый бульвар, 6, Россия)

Автор, ответственный за переписку:
Центер Иосиф Маркович,
e-mail: tsenter1998@mail.ru

РЕЗЮМЕ

Обоснование. *Избыточный вес и ожирение существенно увеличивают риск преждевременной смерти и развития хронических заболеваний. Разработано множество антропометрических индексов, позволяющих верифицировать ожирение, хотя лучший среди них до сих пор остаётся не определённым.*
Цель исследования. *Определить половозрастные особенности динамики антропометрических показателей, характеризующих ожирение.*
Методы. *Программа реализовывалась в период с 2015 по 2020 г. и предусматривала выполнение выборочного исследования. В базовое исследование было включено 1124 женщины и 476 мужчин. Средний возраст составлял 54,9 ± 9,75 и 52,6 ± 10,0 года соответственно (p < 0,001). Для выявления особенностей, связанных с полом, все участники были разделены на три возрастные группы: 35–49 лет, 50–59 лет и 60–70 лет. Период наблюдения составлял 3 года.*
Для определения уровня висцерального жира использовался анализатор жировой массы BC-532 (Tanita Health Equipment HK Ltd., Гонконг). Также рассчитывались индекс массы тела (ИМТ), индекс «талия – бёдра» (ОТ/ОБ, окружность талии/окружность бёдер), индекс висцерального ожирения (ИВО). Статистическая обработка результатов проводилась при помощи программы Statistica 6.0 (StatSoft Inc., США).
Результаты. *Новые случаи ожирения развились у 30,6 % обследованных. Наблюдалось увеличение распространённости ожирения по критериям ОТ (на 8,9 %) и уровень висцерального жира (УВЖ; на 5,4 %) и снижение числа лиц, имеющих ожирение по ОТ/ОБ, на 4,2 %. Из всех показателей только ИВО продемонстрировал статистически значимое снижение средних значений за наблюдаемый период, в то время как ИМТ, ОТ и УВЖ – увеличение.*
Заключение. *Необходимо применение различных критериев для диагностики ожирения, так как отдельные индексы не способны в полной мере отразить половозрастные особенности распределения жира в организме.*

Ключевые слова: *ожирение, антропометрия, эпидемиология, диагностика*

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INTRODUCTION

Worldwide, the prevalence of overweight and obesity is high and continues to rise steadily. While approximately 13.0 % (650 million) of adults worldwide were obese in 2016 [1, 2], by 2030, experts estimate that this figure will be as high as 20.0 % [3]. This condition is known to significantly increase the risk of developing a large number of chronic diseases, including metabolic, cardiovascular, musculoskeletal, neurodegenerative and psychiatric diseases, as well as several forms of cancer [4]. Abdominal obesity is an independent risk factor for metabolic and cardiovascular disease and mortality [5]. Obesity-related noncommunicable diseases account for more than 5 million deaths worldwide each year, with more than half occurring in people under the age of 70 years [6]. Additionally, obesity is a major cause of reduced quality of life, disability and social disadvantage, and it is closely associated with various social factors [3, 7].

Currently, body mass index (BMI) and waist circumference (WC) are still considered the main epidemiological indicators of general and abdominal obesity [8]. However, their usefulness is diminished by the inability to account for body fat distribution [9]. Differences in adipose tissue distribution may contribute to heterogeneity in the clinical and biological manifestations of obesity. Several anthropometric indices have been developed specifically to describe fat distribution, including waist-to-height ratio, BMI, visceral adiposopathy index (VAI), and visceral fat level (VFL). Some studies were reported that waist-to-height ratio was a better predictor of arterial hypertension, diabetes mellitus and hyperlipidaemia than BMI and WC. Body shape index was a significant risk factor for premature mortality in the general population. Some studies have demonstrated that VAI is superior to BMI and WC in predicting arterial hypertension [10]. However, the best obesity verification index that predicted or was closely related to metabolic factors is still controversial and inconclusive [8, 11].

THE AIM

To determine sex and age peculiarities of the anthropometric indicators dynamics which characterise obesity.

MATERIAL AND METHODS

The research program has been organised and implemented between 2015 and 2020. It involved performing a sample survey, for which groups of respondents aged 35–70 were formed. All surveys were conducted in compliance with the "Ethical Principles for Medical Research Involving Human Subjects" in accordance with the "Rules of Clinical Practice in the Russian Federation". The study record was approved by the local ethical committee of Research Institute for Complex Issues of Cardiovascular Diseases (Minutes No. 7 as of 2015). Patients signed informed consent in the prescribed form prior to inclusion in the study. The representativeness of the sample was ensured by random selection in three consecutive stages using the Kish method. Inclusion criteria: age between 35 and 70 years inclusive; stable residence in the selected area for the next 4 years. Individuals under 35 and over 71 years of age, as well as respondents planning to move from their chosen residence in the next 4 years who declined to participate, were not included in the study.

A total of 1124 women and 476 men were included in the pivotal study. The median age for 444 males was 53.5 (44–61) years and for females 57.0 (47–63) years ($p < 0.001$); therefore, all respondents were divided into three age groups to identify sex-age differences: 35–49 years, 50–59 years, 60–70 years. Follow-up lasted 3 years from the first visit. However, during COVID-19 pandemics, it was not possible to adhere to the surveillance time frame due to restrictions on medical preventive examinations. Finally, the prospective phase ended with 60.0 % of participants from each age group at baseline who had a follow-up period of 3 years or less visiting the research centre. As the project progressed, it was found that 807 individuals had follow-up dates that met the above inclusion criteria (84.1 % response rate). Meanwhile, 44 individuals died, 32 moved to another location, and 157 individuals refused further participation in the study; 731 individuals completed the entire list of surveys.

Visceral fat levels were determined with a BC-532 fat mass analyzer (Tanita Corporation, Japan). A level of 1 to 12 conventional units was defined as a healthy level of visceral fat; 13 to 59 conventional units was defined as an elevated level. BMI, waist-to-hip ratio (WC/HC (hip circumference)) were determined using traditional formulas. Visceral adiposopathy index (VAI) was calculated using the formulas:

$$\text{in men: } VAI = WC / (39.68 + (1.88 \times BMI)) \times (TG / 1.03) \times (1.31 / HDL)$$

$$\text{in women: } VAI = WC / (36.58 + (1.89 \times BMI)) \times (TG / 0.81) \times (1.52 / HDL),$$

where: TG – triglycerides; HDL – high-density lipoproteins.

Statistical processing of the obtained data was performed using Statistica 6.0 software (StatSoft Inc., USA) (License No. AXXR003E608729FAN10 dated March 31, 2010). Quantitative variables are presented as median (*Me*) and percentiles (25 %–75 %), while qualitative attributes are presented as frequencies (percentages). The Wilcoxon test was used to compare quantitative variables (baseline and prospective measures); Pearson's Chi-square test was used to compare qualitative variables. The critical level of significance was ≤ 0.05.

RESULTS

The incidence of obesity among women at pivotal study varied from 20.9 % by VFL to 76.5 % by WC, among men from 33.4 % (VAI) to 73.9 % (WC/HC). The prevalence

of obesity by the selected criteria has been summarised in more detail by the authors previously [12]. One should be mentioned that in a comparative analysis, among all the analysed parameters characterising the presence of obesity, only VAI showed a statistically significant decrease in mean values over the observed period, while BMI, WC/HC, WC and VFL increased (Table 1).

In the prospective stage of the study, 247 new cases of obesity were diagnosed (30.6 % of the surveyed population). Sex differences were determined using WC/HC and VFL criteria. Specifically, men were more likely than women to be diagnosed with an increase in this condition by WC/HC (64.9 % vs. 35.1 %, respectively; $p < 0.001$). Meanwhile, an increase in the proportion of VFL obese individuals was recorded more in women than in men (53.7 % and 46.3 %, respectively; $p = 0.003$). The differences did not reach statistical significance for the other parameters studied: for BMI, new cases of obesity were diagnosed in 26.8 % of men and 73.2 % of women ($p = 0.617$); for WC, obesity was almost twice as common in women as in men (65.4 % vs. 34.6 %, respectively; $p = 0.382$); new cases of obesity according to VAI were recorded in 26.6 % of males and 73.4 % of females ($p = 0.494$). Statistically significant age differences were revealed only in the case of WC criterion: the maximum increase of this indicator was observed in the group of 50–59 years – 47.4 %, in the younger age group it was 35.9 % and the minimum increase was observed in the group of 60–70 years (16.7 %) ($p = 0.001$). It was also observed that the maximum prevalence of obesity according to BMI, VFL and VAI criteria was in the age group of 60–70 years (36.6 %, 44.8 % and 40.6 % respectively) and according to WC/HC criteria in the age group of 35–49 years (40.3 %).

Between 2015 and 2019, there was a decrease in the prevalence of obesity assessed by WC/HC from 74.6 % to 70.4 % ($p = 0.034$) (Fig. 1). An increase of 8.9 % ($p < 0.001$) and 5.4 % ($p = 0.010$) in the detection rate of this pathology was observed as per WC and VFL criteria, respectively.

When analysing the sex- and age-specific dynamics of obesity assessed by various criteria, statistically significant differences were revealed when studying WC and WC/HC parameters (Table 2). Specifically, the prevalence of abdominal obesity in young women increased by 11.8 %. Furthermore, both sexes aged 50–59 years also showed an increase in the incidence of obesity detection by this criterion (by 13.9 % and 12.3 %, respectively). An interesting pattern was observed when examining

TABLE 1

DYNAMICS OF OBESITY RATES DURING THE STUDY PERIOD, Me (25 %–75 %)

Indicators	Basic stage	Prospective stage	*p* value
Body mass index	29.0 (25.2–33.1)	29.3 (25.8–33.2)	< 0.001
Waist-to-hip ratio	0.88 (0.8–0.9)	0.92 (0.8–1.0)	< 0.001
Waist circumference	93.0 (83.0–103.0)	98.0 (88.0–108.0)	< 0.001
Visceral fat level	10.0 (7.0–13.0)	11.0 (8.0–14.0)	< 0.001
Visceral adiposopathy index	1.58 (0.9–2.6)	1.47 (0.9–2.4)	0.001

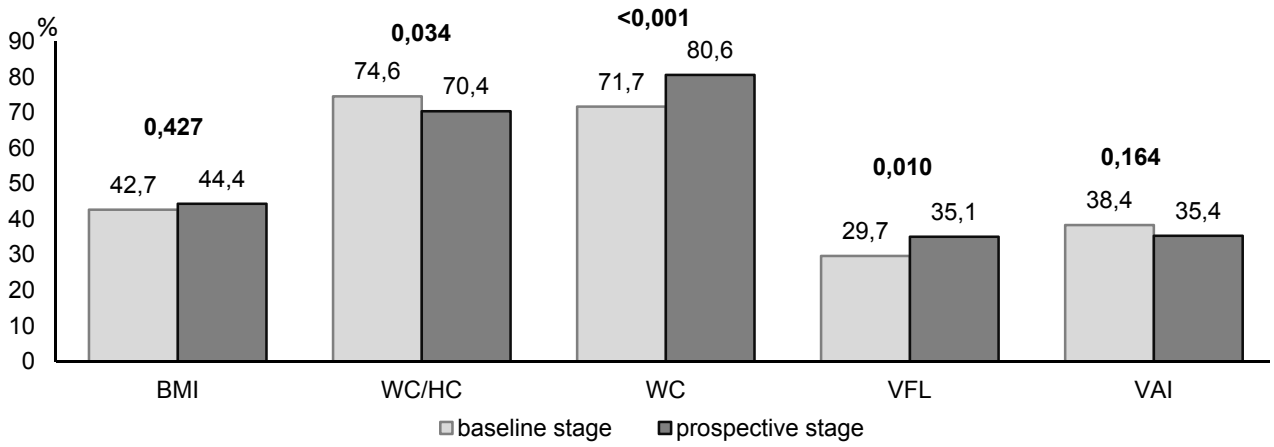

FIG. 1.

Change in the obesity prevalence diagnosed by different criteria (2015–2019)

TABLE 2

GENDER AND AGE-SPECIFIC DYNAMICS OF OBESITY ASSESSED BY DIFFERENT ANTHROPOMETRIC INDICATORS (%)

Age, years	Sex	BMI		*p* value	WC		*p* value	WC/HC		*p* value	VFL		*p* value	VAI		*p* value
		B	P		B	P		B	P		B	P		B	P	
35–49	Male	33.7	45.1	0.093	53.9	62.0	0.248	64.0	77.5	0.041	28.6	39.4	0.099	33.1	40.8	0.251
	Female	27.5	29.9	0.605	55.9	67.7	0.021	53.5	39.4	0.007	3.0	3.1	0.943	27.8	22.8	0.281
50–59	Male	37.3	31.3	0.353	60.8	74.7	0.030	76.6	89.2	0.018	51.9	62.6	0.110	39.9	33.7	0.350
	Female	47.9	49.2	0.775	76.6	88.9	0.001	75.5	63.0	0.002	21.4	23.8	0.530	43.7	42.0	0.704
60–70	Male	37.1	38.8	0.817	67.9	70.1	0.740	83.6	91.0	0.056	77.1	80.6	0.573	26.4	14.9	0.065
	Female	57.2	56.5	0.861	91.9	94.0	0.339	90.3	79.5	0.0002	33.9	37.5	0.381	47.5	43.0	0.288

Note. B – baseline stage; P – prospective stage.

trends in the prevalence of obesity by WC/HC criterion: in females, there was a decrease regardless of age group (by 14.1 %, 12.2 % and 10.8 %, respectively). Meanwhile, in men, an increase in the prevalence of obesity was revealed at the ages of 35–49 and 50–59 years (by 13.5 % and 12.6 %, respectively).

DISCUSSION

The results of a single-centre, three-year study revealed an increase in the prevalence of obesity by WC and VFL criteria and a decrease by WC/HC criteria. The sex- and age-specific dynamics of obesity according to the studied criteria consisted in an increase in the prevalence of abdominal obesity in women of 35–59 years old, but a decrease in the frequency of detection in all age groups according to the WC/HC criterion. In males, there was an increase in the detection of obesity by WC at age 50–59 years and by WC/HC at age 35–59 years. The median values of all studied indicators (except VAI), however, statistically significantly increased. This fact evidences the need to apply different criteria for the diagnosis of obesity, as BMI is probably not able to fully reflect the sex- and age-specific distribution of body fat [13].

The increasing prevalence of obesity has been observed in most countries of the world during the last decades. For instance, in a study of the Korean National Health Insurance Service's national health check-up database, the prevalence of obesity increased steadily over a 10-year period from 2009 (29.7 %) to 2018 (35.7 %) among the entire population and in all age groups. The prevalence of abdominal obesity has also increased, from 19.0 % in 2009 to 23.8 % in 2018. The increase in the prevalence of abdominal obesity was most evident in men (from 20.7 % to 28.1 %, respectively). In women, the prevalence of abdominal obesity increased from 16.2 % to 18.2 % overall, but decreased between the ages of 50–60 years [14].

Among 20 populous countries, Egypt had the highest adult obesity rate in 2015 with a rate of 34.9 %, while Vietnam had the lowest with a rate of 1.6 %. The prevalence of obesity more than doubled in 13 of these 20 countries between 1980 and 2015, and only the Democratic Republic of Congo did not show an increase in this pathology [15]. In Africa, between 1980 and 2014, the age-standardized mean BMI increased from 21.0 kg/m^2 (95 % confidence interval (95 % CI): 20.3–21.7) to 23.0 kg/m^2 (95 % CI: 22.7–23.3) in men and from 21.9 to 24.9 kg/m^2 in women [16]. There are few large epidemiologic studies in the Russian Federation. The study of obesity in the regions of the country is mostly one-sided and ignores the full range of factors affecting the incidence of these pathologies. There is currently a low efficiency in the diagnosis and treatment of obesity [17]. According to the study "Epidemiology of cardiovascular diseases and their risk factors in the Russian Federation" (ESSE-RF), the prevalence of obesity reached 29.7 % [18]. Meanwhile, in different regions of the Russian Federation, this indicator varies from 22.5 % to 44.5 % for BMI and from 43.0 % to 67.0 % for WC. Abdominal obesity was more closely associated with high risks of cardiovascular disease (CVD) and diabetes mellitus and had a significantly higher prevalence of 55.0 % vs. 33.4 % for BMI [19].

If the above trends in the prevalence of obesity persist, the chances of stabilising body weight in the general population are nil [20]. Scientists estimate that in the next five years, the global prevalence of obesity will reach 18 % and 21 % in men and women, respectively [20].

Overall, the prevalence of obesity and overweight in Middle Eastern countries remained stable from 2000 to 2020 with an average prevalence of 23 %. The prevalence of overweight, however, decreased from 34.8 % (95 % CI: 32.4–37.4) to 32.8 % (95 % CI: 31.4–34.4) over these time intervals. Meanwhile, in women, the prevalence of obesity and overweight decreased from 26.6 % (95 % CI: 22.9–30.9) and 32.3 % (95 % CI: 29.8–35.0) between

2000 and 2006 to 23.1 % (95 % CI: 20.8–25.7) and 32.8 % (95 % CI: 31.39–34.38) between 2014 and 2020, respectively. In contrast, the prevalence of obesity in men increased from 20.1 % (95 % CI: 16.24–24.82) from 2000 to 2006 to 23.5 % (95 % CI: 20.3–27.2) from 2014 to 2020. Although, the prevalence of overweight in males remained stable (39.0 %) during these periods [21].

The rate of obesity increase in Europe is higher in men than in women (3.1 % vs. 1.9 % per year). If the increase rate remains at the estimated level in 2030, Poland is likely to have more obese men (38.1 %) than women (32.7 %) and Europe will have 36.6 % and 32.0 %, respectively [22].

According to these trends, the main driving force behind the obesity epidemic, according to the authors, is the westernisation of lifestyles in countries [23, 24]. Policies implemented in society should promote behavioural change at the personal level with a focus on increasing consumption of healthy diet and physical activity [15]. However, the heterogeneity in obesity rates between countries may mean that social and other factors and their differences are associated with obesity status. Increases in obesity have more often been accompanied by improvements in the economy, especially in a number of developing countries [15].

The foregoing data suggest that there are inconsistencies in the assessment of current anthropometric indexes, and they cannot provide a comprehensive prediction of metabolic risk factors. Consequently, further studies should be conducted to elucidate the association of anthropometric parameters with cardiovascular risk factors [25, 26].

CONCLUSIONS

New cases of obesity were identified in 30.6 % of respondents during the period analysed: an increase in prevalence was observed using WC and VFL criteria (by 8.9 % and 5.4 %, respectively). While at the same time, the WC/HC criterion showed a 4.2 % decrease in the number of persons with this pathology. The median of all studied indicators (except VAI) increased statistically significantly over the three years of follow-up. Men were more likely than women to be diagnosed with an increase in this condition by WC/HC (64.9 % vs. 35.1 %, respectively; $p < 0.001$). Meanwhile, an increase in the proportion of VFL obese individuals was recorded more in women than in men (53.7 % and 46.3 %, respectively; $p = 0.003$). Age differences were only observed when the WC criterion was applied: the maximum increase was observed at 50–59 years (47.4 %) and at 35–49 years (35.9 %), while the minimum increase was observed at 60–70 years (16.7 %; $p = 0.001$). The maximum prevalence of obesity according to BMI, VFL and VAI criteria was in the age group of 60–70 years (36.6 %, 44.8 % and 40.6 %, respectively) and according to WC/HC criteria in 35–49 years (40.3 %). The choice of the best method to measure obesity for predicting CVD risk factors remains controversial. Further studies are required in populations for which the various anthropometric measures have not been thoroughly analysed and compared. A comprehensive approach to diagnosis is required, considering traditional, socio-economic and behavioural factors specific to a particular region. Targeting obesity risk reduction will help reduce the burden of circulatory disease in the adult population.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Daria P. Tsygankova – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: cigadp@kemcardio.ru, https://orcid.org/0000-0001-6136-0518

Evgeny D. Bazdyrev – Dr. Sc. (Med.), Head of the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: bazded@kemcardio.ru, https://orcid.org/0000-0002-3023-6239

Olga V. Nakhratova – Junior Research Officer at the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: nahratovao.v@yandex.ru, https://orcid.org/0000-0002-2778-6926

Iosif M. Tsenter – Clinical Research Assistant at the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: tsenter1998@mail.ru, https://orcid.org/0000-0002-9048-8294

Timur F. Gaziev – Junior Research Officer at the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: gaziev.tim@yandex.ru, https://orcid.org/0000-0003-3840-744X

Elena V. Indukaeva – Cand. Sc. (Med.), Senior Research Officer at the Laboratory of Cardiovascular Diseases of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: indev@kemcardio.ru, https://orcid.org/0000-0002-6911-6568

Galina V. Artamonova – Dr. Sc. (Med.), Professor, Head of the Department of Optimization of Medical Care for Cardiovascular Diseases, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: artamonova@kemcardio.ru, https://orcid.org/0000-0003-2279-3307

Olga L. Barbarash – Dr. Sc. (Med), Professor, Member of the RAS, Director, Research Institute of Complex Problems of Cardiovascular Diseases, e-mail: barbol@kemcardio.ru, https://orcid.org/0000-0002-4642-3610

PSYCHOLOGY AND PSYCHIATRY

INDIVIDUAL VARIABILITY OF HIGHER MENTAL FUNCTIONS IN PRESCHOOL CHILDREN WITH REGARD TO THE MATERIAL PROSPERITY OF THE FAMILY (NEUROPSYCHOLOGICAL ANALYSIS)

Nerovnykh M.S.

Katanov Khakass State University (Lenina Ave. 90, Abakan 655017, Russian Federation)

Corresponding author:
Marina S. Nerovnykh,
e-mail: s.mariana@list.ru

ABSTRACT

The problem of human individual development requires not just the accumulation and generalization of data, but also clarification, a systematic understanding of the individual variability of higher mental functions in relation to environmental factors and taking into account the risks associated with their formation.

The aim of the study. *To identify systematic patterns of correlation between individual variability of higher mental functions of preschool children and the material prosperity of their families.*

Materials and methods. *Traditional neuropsychological tests developed by A.R. Luria and adapted in the neuropsychology laboratory of the Faculty of Psychology at Lomonosov Moscow State University were used. We examined 180 preschool children from families with high, average, and low income.*

Results. *The greatest individual variability in the period of preschool age in relation to the level of material prosperity of the family, are the functions with a long period of formation, these are functions of block III of the brain, and the functions provided mainly by the left hemispheric parts of the brain. Children from the most affluent families have the highest indices of brain block III functions ($p < 0.001$) and left hemispheric functions ($p < 0.001$). Preschoolers from low-affluence families had indexes of both front brain function ($p < 0.001$) and left hemispheric function ($p < 0.001$) in the zone of negative values.*

Conclusion. *The empirical results of the study allow us to clarify that the factor of material prosperity of the family, both directly, factor-wise, and indirectly, cumulatively, through the system of proximal factors, can make its selective contribution to the variability of indicators of children's higher mental functions.*

Key words: *higher mental functions, material prosperity, preschoolers, variability, socio-demographic characteristics, neuropsychological analysis*

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ИНДИВИДУАЛЬНАЯ ИЗМЕНЧИВОСТЬ ВЫСШИХ ПСИХИЧЕСКИХ ФУНКЦИЙ ДОШКОЛЬНИКОВ В АСПЕКТЕ МАТЕРИАЛЬНОГО ДОСТАТКА СЕМЬИ (НЕЙРОПСИХОЛОГИЧЕСКИЙ АНАЛИЗ)

Неровных М.С.

ФГБОУ ВО «Хакасский государственный университет им. Н.Ф. Катанова» (655017, г. Абакан, просп. Ленина, 90, Россия)

Автор, ответственный за переписку:
Неровных Марина Сергеевна,
e-mail: s.mariana@list.ru

РЕЗЮМЕ

Решение проблем индивидуального развития человека требует не просто накопления и обобщения данных, но и уточнения, системного понимания индивидуальной изменчивости высших психических функций в соотношении с факторами среды и учёта рисков, сопутствующих их формированию.

Цель исследования. *Выявить системные закономерности соотношений индивидуальной изменчивости высших психических функций детей дошкольного возраста с материальным уровнем их семей.*

Методы. *Применялись традиционные нейропсихологические пробы, разработанные А.Р. Лурией и прошедшие адаптацию в лаборатории нейропсихологии факультета психологии МГУ им. М.В. Ломоносова. Было обследовано 180 детей дошкольного возраста из семей с высоким, средним и низким уровнем доходов.*

Результаты. *Наибольшей индивидуальной изменчивостью в период дошкольного возраста в соотношении с материальным уровнем семьи подвержены функции с долгим периодом формирования – это функции III блока мозга, и функции, обеспечиваемые преимущественно левополушарными отделами головного мозга. Дети из наиболее обеспеченных семей имеют самые высокие показатели индексов функций III блока мозга ($p < 0,001$) и левополушарных функций ($p < 0,001$). У дошкольников из семей низкого материального уровня в зоне отрицательных значений оказались индексы функций как передних отделов головного мозга ($p < 0,001$), так и функций левополушарных отделов ($p < 0,001$).*

Заключение. *Полученные эмпирические результаты исследования позволяют уточнить, что фактор материального достатка семьи – как непосредственно, факторно, так и опосредованно, кумулятивно, через систему проксимальных факторов, – может вносить свой избирательный вклад в изменчивость показателей высших психических функций детей, что проявляется в более интенсивном темпе формирования одних групп функций и менее интенсивном – других, а также в выборе ведущей стратегии обработки информации, опирающейся на активность левого или правого полушария.*

Ключевые слова: *высшие психические функции, материальный уровень, дошкольники, изменчивость, социально-демографические характеристики, нейропсихологический анализ*

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INTRODUCTION

Since the second half of the 19th century, the focus of the central problems of psychology has been devoted to the study of the sources of individual variations in mental processes. It has been revealed that individual differences are in a complex relation of the complementary action of heredity and environment, as well as the activity of the individual himself. Heredity allows to ensure the stability of psychological properties of biological species, to transmit individual traits to the next generation; the environment provides variability of individual traits and the ability to adapt to changing conditions of life [1]. At the same time, heredity allows a very wide range of variations in the path of ontogenetic development, which are the result of various reactions of the organism – biochemical, physiological, psychological [2]. An obvious insight in this research context is the understanding that changing environments unfold the innate programmes of human manifestation in different ways. Along with this, in the process of development of human individuality there are areas more variable and sensitive to environmental influence, and relatively stable.

The functional brain activity organisation is one of the most important individual properties of a human being, objectively formed at the early stages of ontogenesis. Functional systems have a lifetime path of formation, are transformed in the process of realisation of a particular mental activity and are conditioned by social experience [3]. It is the early experience that largely determines which of the possible trajectories a person's developmental path will follow. The theory of "afferent field", introduced by P.K. Anokhin reveals the idea that a functional system possessing a certain complex of afferent impulses has a significant degree of maturity already in the period of early ontogenesis, and for a number of systems – already in the embryonic period [4]. At the same time, the afferentations included in the activity of a functional system, which are not involved in the process of realisation of a particular mental activity, begin to narrow down and pass into a latent state; meanwhile, only a small circle of active afferentations remains relevant [5].

Consequently, in the process of ontogenetic development, functional systems undergo intensive changes, as a result of which the same tasks are being implemented by completely different means, and the choice of strategies for solving tasks is conditioned by the factors of the child's social environment.

Considering that the social environment of early childhood is primarily the family, it is necessary to know which family environmental variables are of primary importance in the functional development of the child and what is the nature of their impact over the course of development. Having analysed scientific literature, it can be concluded that the variability of the morphofunctional state of the child's brain has a significant relationship with the socio-demographic characteristics of the family, including its integral component – socio-economic status [6, 7].

In considering the problems related to the socio-economic status of the family, two opposite strategies of its examination can be found in foreign studies. The first approach considers socio-economic status as a set of several interrelated factors combined into a common index (education, parents' profession, material prosperity) [6]. A different research strategy is presented by the authors [8], who insist that the relationship between these two variables (income and education), is not rigidly deterministic. For example, higher levels of education are associated with more favorable economic opportunities, including through higher income, more extensive social and psychological resources (higher social support, higher levels of control). That said, there are examples of people who are educated but earn relatively little; there are also reverse examples of people with no education who have achieved financial prosperity. Thus, different indicators of socio-economic status are not interchangeable, so there is a need to measure separate components of socio-economic status and separately assess the contribution of each of them – this approach gives a more detailed picture of the social status of a particular individual.

In our opinion, insufficient attention is paid to the study of the influence of such socio-demographic parameters of the family as its material status on the process of development of higher mental functions. The published data related to the problem under consideration are presented to a large extent in the works of foreign authors; there is a certain empirical deficit in Russian studies. It is important to note that this dependence (of economic status and individual differences) is not direct but mediated. The available literature notes that families with different material levels are characterized by specific environmental conditions, which affects various aspects of family functioning and, consequently, the process of ontogenetic development [9]. A certain part of studies [10, 11] points to unequal access to material and cultural resources to stimulate children's cognitive development, including through the opportunity to purchase a variety of educational games, books, joint leisure activities (visiting the theatre, museums, libraries, exhibitions, travel), etc. Moreover, as some authors note [12], there are significant differences between families with different economic status in the ability to give their children a continuous and quality education. D.N. Chernov [13] emphasizes that if parents have difficulties in the implementation of educational and developmental functions, they begin to "include" in these processes such social institutions as the system of additional education, hobby groups, music, art, sports schools, sections, etc., which, according to the author, partially compensates for the lack of parental attention.

An important aspect of a child's development is his or her language environment. The process of a child's language socialization occurs through the natural inclusion of the child in the community spoken by his or her immediate environment. Thus, being oriented to the style preferences of the speakers of a language variant (dialect), it internalizes social standards and ideas about de-

sirable forms of communication [14]. The relationship between the volume of the child's vocabulary, the complexity of grammaticalization of utterances and various components of socio-demographic parameters of the family, such as income, parents' education, their profession, and quality of care, has been confirmed by empirical material from many studies [15, 16].

A cycle of research is devoted to the study of the psychological climate of families with different economic conditions. The authors note that the effect of emotional stability, sensitivity to the child's needs can be conditioned by the social position of parents [17]. For instance, the effect of emotional and psychological tension in low-income families due to financial instability, inconsistent employment of parents has been revealed, which in turn affects the quality of parental behavior [18]. Other studies have concluded that low family socio-economic status is associated with the presence of chronic stress and co-occurring anxiety [19]. Researchers have concluded that during critical periods of brain maturation, stressful conditions can have a "programming" effect and lead to irreversible and long-lasting changes [20]. Therefore, the effect of the stress response arising as a result of the penetration of various pathogens into the organism leads to a "programming" effect on the development of the brain. A pronounced stress response resulting from the administration of bacterial lipopolysaccharide to baby rats led to significant rearrangements in the central nervous system. The outcome of such stressful influence in the early postnatal period is the manifestation of behavioural disorders in sexually mature individuals [21]. The most specific consequences of such stress exposure in rodents include impaired stress tolerance, symptoms of anxiety, depressed state, decreased cognitive activity, and disorders of neuroplasticity processes that underlie learning and memory formation [22, 23]. The authors extrapolate that these kinds of stressors include disruption of their parental care of offspring, including maternal depression, changes in feeding patterns, and emotional upheaval [24].

Numerous data have been accumulated that suggest a strong correlation between anthropometric indicators of the newborn and the socio-economic status of the mother [25]. According to Yu.E. Veltischev [26], lagging anthropometric indicators of newborns (low weight, small head volume) are significantly common in families with insufficiently high socio-economic level. Low birth weight has long-term effects on mental and physical development in all subsequent stages of ontogenesis. Such consequences include, for example, problems in the formation of spatial functions and programming as well as control functions at school age [27]. There is a consensus of researcher's opinion that it is not the poor financial situation of the family itself that leads to poorer development, but a number of concomitant circumstances.

This analysis of the sources of the problem under study is not limited to this. Additionally, there are studies that consider the amount of time spent with the child and the number of books read, value orientations and attitudes, nutritional quality, living conditions [28], and many others as the main mechanisms of mediating the impact of family material prosperity in children's morphofunctional development.

Despite the fairly well-developed problem of explanatory mechanisms that influence the considered factors in children's intellectual and physical development and their performance in school, there are few studies devoted to children's neurocognitive development in our country. Essential is the understanding that the problem is interdisciplinary: individual differences of children in relation to material prosperity of the family are recognised as an acute problem from the point of view of general psychology, neuropsychology of individual differences, psychophysiology, paediatrics, sociological and economic sciences.

Therefore, the theoretical analysis of the problem made it possible to define the purpose of the study – to identify systemic regularities of correlations between individual variability of higher mental functions of preschool children and the material level of their families.

METHODS

Traditional neuropsychological tests developed by A.R. Luria [29] and adapted in the Laboratory of Neuropsychology of the Faculty of Psychology at Lomonosov Moscow State University were included in the study. Statistical processing of the data was performed using Univariate Analysis of Variance, with pairwise multiple comparisons with Scheffé correction. Data processing was performed using the IBM SPSS Statistics 26 software package (IBM Corp., USA).

180 preschool children were examined, the average age was 6.5 years. Neuropsychological diagnostics was carried out individually with each child. The duration of the examination was 55–70 minutes.

Distribution of children into groups was carried out by means of stratometric modeling strategy of the sample – with the help of a specially designed questionnaire. Strata were material level of the family: high level of income (more than two subsistence minimums per family member) – 26 % of families; average level of income (from one to two subsistence minimums per family member) – 35 % of families; low level of income (less than one subsistence minimum per family member) – 39 % of families.

The methodology of families (respondents) distribution by income level is based on the normative criterion. The classification of population groups by income, developed by experts of the All-Russian Center for Living Standards, is taken as a basis: the least well-off (low-income), well-off below the average level, middle-income, high-income [30]. In contrast to this classification, we have identified three groups of families by income level – low, average and high. The average-income family group combined lower-middle-income and average-income families.

The criterion for assignment to a certain group are social standards – subsistence minimum, socially accept-

able (restorative) consumer budget, consumer budget of average income and consumer budget of high income [30]. When classifying families to the low-income group, we follow the absolute monetary approach (households with average per capita monetary incomes below the subsistence minimum are recognized as poor). As a threshold value separating the group of families with low and average income, the value of two subsistence minimums is adopted, since the socially acceptable (recovery) consumer budget is not less than 2–2.5 subsistence minimums.

All children were pupils of municipal budget preschool educational organizations. The following facts were the criteria for selection of the participants: absence of diagnosed neurological disorders, cerebral-organic pathology, and developmental deviations. All lived in a two-parent full family where they were the only child (42 %), or had one sibling (51 %), less frequently – two siblings (7 %). The sample population, allocated with regard to stratification of family material status, was equalized by sex of children (50 % of boys and 50 % of girls, respectively). Mothers of 47 % of children have higher education, 36 % have specialized secondary education, and 17 % have secondary education. 31 % of preschool children are brought up by fathers with higher education, 26 % – with specialized secondary education, 42 % – with secondary education.

The analysis of children's higher mental functions was carried out in accordance with the approach proposed by A.R. Luria [3], based on the idea of a structural-functional model of the three brain blocks activity considering interhemispheric asymmetry. To this end, during the processing of the quantitative parameters of the samples, a procedure was performed to calculate generalised (aggregated) indicators – neuropsychological indices, which included the most unambiguously interpretable parameters of performed samples (a total of 122 parameters).

Functional features of movements and speech serial organisation, as well as programming, control and regulation of arbitrary forms of activity are presented in a generalised index – the index of the III block of the brain (cerebrum anterior). This indicator includes a quantitative assessment of test performance: graphomotor coordination, dynamic hand praxis, reciprocal coordination, choice reaction, rhythms according to instructions, syllabic structure of words, text retelling (the criteria of programming and grammatical design of the utterance, as well as the semantic adequacy of the utterance were assessed).

The index of Block II functions reflects the state of kinaesthetic functions (oral and manual praxis), functions of visual-spatial, visual and auditory information processing.

Since Block II of the brain (block of reception, storage and processing of information, posterior cortex of cerebrum) is functionally lateralised, it includes two indices – index of information processing by left-hemispheric and by right-hemispheric type.

The left hemisphere function index (sinistrocerebral index) is represented by quantitative assessments of oral praxis, lexical components of text processing, right hand finger posture praxis, comprehension of complex logical and grammatical constructions, and auditory-verbal memory (short-term and long-term).

The right hemisphere function index (dextrocerebral index) was assessed by the performance of left-hand finger posture praxis, copying a table, recognising undrawn images, and the volume of involuntary auditory-spatial and visual-spatial memory.

The obtained index values have undergone a standardization procedure. Standardized z-scores were calculated using the formula: $z = (x - \mu) / \sigma$. According to the presented formula, the sample mean for this parameter was subtracted from the value of the individual indicator for the parameter, and the obtained result was divided by the standard deviation. The correlation of these indicators made it possible to obtain neuropsychological profiles reflecting the functional state of children's higher mental functions.

RESULTS

The presented standardized indices allow us to see how much and in what (better or worse) direction the individual value of the index of functions of Blocks III or II (left or right hemisphere) of the preschoolers' brain deviates from the average group value (Fig. 1).

The presented indices' values and their deviations from group averages indicate the unevenness in the development of functions of Block III of the brain, as well as the left hemisphere within the population of preschoolers in correlation with the material level of the family. The most stable indices are presented in the right hemisphere function indices.

Functional indices in preschoolers from low-income families are significantly reduced (Block III and left hemisphere index), which can be regarded as a manifestation of a sign of deficit of these functions. It should be emphasised that the relative decrease in the indices is not evidence of fatal impairment of certain functions, but of signs of partial deficiency and insufficiency of their formation to the level of the same-age population.

By applying single factor analysis of variance with pairwise multiple comparisons with Scheffe's correction, the following results were obtained as summarised in Table 1.

Preschoolers from the most affluent families had the highest indices of brain Block III function indices (differences with Group 2 ($p = 0.046$) and Group 3 ($p < 0.001$)) and sinistrocerebral indices (differences with Group 3; $p < 0.001$). Compared to them, preschoolers from average-income families have uniformly stable indicators of all functions, but their index values are lower than those of children who are more successful in functional development. In preschoolers from families of low material prosperity, the function indices of both cerebrum anterior (differences with Groups 1 and 2; $p < 0.001$) and sinistrocerebral indexes (differences with Group 1; $p < 0.001$) were in the range of negative values.

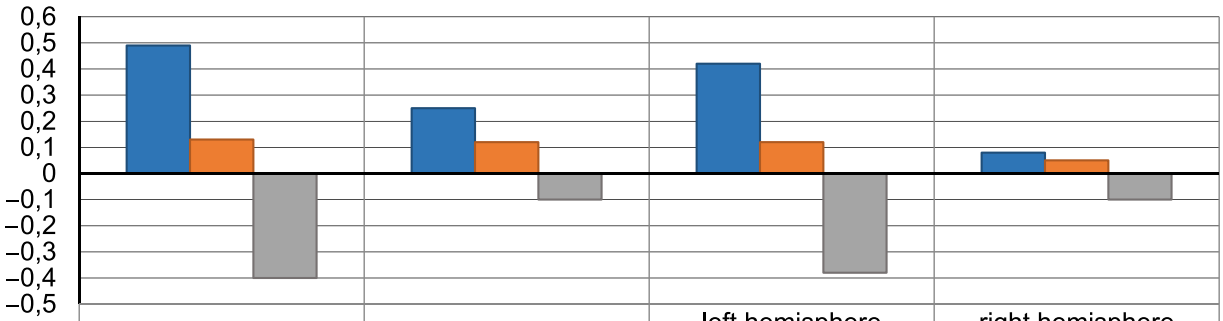


FIG. 1.
Standardized average values of the indices of higher mental functions in preschool children in relation to the level of material prosperity of the family

TABLE 1

LEVELS OF DIFFERENCES IN THE INDICATORS OF THE FUNCTIONS OF PRESCHOOL CHILDREN IN RELATION TO THE LEVEL OF MATERIAL PROSPERITY OF THE FAMILY

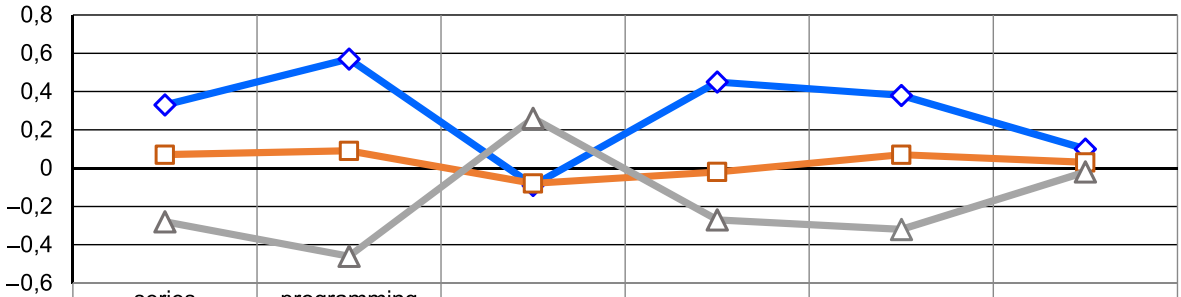
Indicators	Material prosperity (1 – high; 2 – average; 3 – low)				
	Level of difference			ANOVA	
	1–2	1–3	2–3	Sig	F
Brain Block III indices	0.046	< 0.001	< 0.001	< 0.001	15.57
Brain Block II indices	0.050	< 0.001	0.014	< 0.001	9.35
Sinistrocerebral indices of the brain	–	< 0.001	0.002	< 0.001	11.00
Dextrocerebral indices of the brain	–	–	–	–	0.64

The obtained results indicate individual unevenness of children's higher mental functions and require a more differentiated analysis of individual indices of higher mental functions, which will enable us to determine the reason of increased or decreased overall indices of brain block indices and to obtain neuropsychological profiles of preschool children of the selected groups (Fig. 2).

The neuropsychological profiles and the results of the analysis of variance presented in Figure 2 confirm the significant effect of the material prosperity of the family in terms of programming and control functions ($p < 0.001$), serial organisation of movements and speech ($p = 0.004$), visual ($p < 0.001$) and auditory ($p < 0.001$) functions, and kinaesthetic praxis ($p = 0.053$). Less differentiated index scores are presented in the functions of visual-spatial information processing.

Children from high-income families (Group 1) have high indices of function: serial organization of movements (differences with Group 3; $p < 0.001$), arbitrary regulation of activity and speech (differences with Groups 2 ($p = 0.008$) and 3 ($p < 0.001$)), auditory functions (differences with Groups 2 ($p = 0.011$) and 3 ($p < 0.001$)), and visual functions (differences with Group 3; $p < 0.001$). Against the background of high indices of almost all higher mental functions, preschool children from the most affluent families show a decrease in the indices of kinaesthetic information processing functions (differences with Group 3; $p = 0.042$).

Analysis of the results of neuropsychological tests for the study of the functions of serial organisation of movements and speech gives the following picture: preschoolers from high-income families (Group 1) demonstrate advantages in the characteristics of dynamic praxis – this is manifested in a better ability to automate a motor skill ($p = 0.017$), fewer errors in the reproduction of the motor programme and a faster rate of work-



	series organization	programming and control	kinesthetic	auditory	visual	visual-spatial
high (1)	0,33	0,57	–0,09	0,45	0,38	0,1
average (2)	0,07	0,09	–0,08	–0,02	0,07	0,03
low (3)	–0,28	–0,46	0,26	–0,27	–0,32	–0,02

FIG. 2.

Indices of various components of the higher mental functions associated with the functions of blocks III and II of the brain in preschool children from families with high (1), middle (2) and low (3) income levels

in-progress ($p = 0.004$). High indices also differentiate this group of children in the ability of serial speech organisation, namely in grammatical structuring when retelling a text ($p < 0.001$). Children from average-income families (Group 2) have some difficulties in automating the motor skill: in the dynamic praxis test, in most cases, their programme execution varies from individual series – "packs" – to smooth change of programme elements (differences with Group 1; $p = 0.040$). However, they make no errors whatsoever in the execution of the programme, or there are isolated difficulties associated with the violation of the movement sequence. The arising difficulties in children of this ontogenesis norm are also connected with the grammatical design of the utterance: in retelling, the text is most often composed in a rather monotonous way, without using complex sentences or with violation of word order (differences with Group 1; $p = 0.006$). Children from low-income families (Group 3) are less efficient in learning and automatising motor skills (differences with Groups 2 ($p = 0.040$) and 1 ($p = 0.005$)) and have the lowest indices of speech utterance serial organisation (differences with Groups 2 ($p = 0.006$) and 1 ($p < 0.001$)), mainly associated with the presence of single non-coarse agrammatisms or paragrammatisms.

Sample analysis of the programming and control function tests showed that children from families with high material prosperity demonstrated higher speed characteristics of the choice reaction (differences with Group 3; $p < 0.001$). In goal-directed activities programmed by verbal instruction, they have the highest productivity (differences with Groups 2 ($p = 0.019$) and 3 ($p = 0.001$)). Group 1 children were also more successful in constructing the semantic programme of an utterance ($p < 0.001$); they made fewer errors caused by inertia. Their productivity of free association actualisation is more productive (differences with Groups 2 ($p = 0.04$) and 3 ($p = 0.04$)). Children from average-income families show relative weakness of functions of activity arbitrary regulation, associated mainly with programming of speech utterance (differences with Group 1; $p = 0.001$) and actualisation of free associations (differences with Group 1; $p = 0.04$) with rather stable high indicators in the choice reaction test. The results of all samples measuring arbitrary regulation of activity and speech by children from the least affluent families indicate insufficient involvement of prefrontal mechanisms of attention to stimulus presentation (differences with Groups 2 ($p = 0.002$) and 1 ($p = 0.001$)), as well as in sentence and text construction (differences with Groups 2 ($p = 0.004$) and 1 ($p < 0.001$)).

Individual differences in kinaesthetic praxis condition were caused by higher productivity of left-handed finger poses by preschoolers from low-income families (differences with Group 1; $p = 0.042$).

Auditory-verbal functions in children from maximally affluent families are characterised by high amounts of involuntary (differences with Group 3; $p = 0.050$) and arbitrary short-term memory (differences with Group 3; $p = 0.001$), as well as delayed reproduction and resistance to interference (differences with Group 3; $p < 0.001$). Children from average-income families are comparable to those from materially prosperous families in terms of auditory-verbal memory capacity, but errors (verbal substitutions based on semantic proximity) indicating difficulties in the left-hemispheric strategy of information processing are quite frequent among them (differences with Group 1; $p = 0.039$). Deterioration of auditory information processing in the preschoolers from low-income families occurs as a result of a decrease in memory indices of this modality (differences with Groups 2 ($p = 0.004$) and 1 ($p = 0.001$)) and, in addition, an increase in word distortions during their reproduction (differences with Groups 1 and 2; $p < 0.001$), an increase in the number of errors associated with verbal substitutions based on semantic proximity (differences with Group 1; $p = 0.032$).

The visual functions of preschoolers from families with a high material level of family are character-

ized by the predominance of analytical, left-hemispheric strategy of visual information processing, which is well traced in the productivity of samples on identification of crossed-out (differences with Group 3; $p = 0.011$) and superimposed images (differences with Group 3; $p < 0.001$). Children from average-income families have the same productivity of recognising perceptually complex images as the preschoolers from maximally affluent families, but they more often make verbal-perceptual errors in the form of forgetting exact names and using semantically similar words (differences with Group 1; $p = 0.040$), which is an indicator of left-hemispheric complexities. Preschoolers from the least financially affluent families have difficulties recognising both crossed-out (differences with Group 1; $p = 0.011$) and superimposed images (differences with Group 1; $p < 0.001$). In addition, there was an increase in verbal-perceptual errors (differences with Group 1; $p = 0.020$).

Indicators of visual-spatial functions in preschool children in general have low values and do not differentiate the preschoolers by the level of their formation, but some components of these functions (projection representations) in children from families with a high material level of family tend to decrease. Since preschool age is not a sensitive period for the formation of these functions, children of this age are characterised by a low level of their formation. An intensive leap in their development should be expected at a later age in the process of purposeful learning.

DISCUSSION

As to the results obtained in general, it should be noted that the empirical data of this study suggest that a significant proportion of the environmental dispersion (from 6 to 23 %) of individual differences in the development of structural components of children's higher mental functions is determined by the factor of family affluence. Individual variability of preschool children's mental functions in relation to the level of family affluence is associated with the preconditions of a more accelerated rate of development of some functions, associated with the risk of "weakening" or "stealing" of other developing functions or with a significant decline in functional capabilities.

The most dynamic restructuring of functional systems occurs in the sensory periods of development and leads to significant changes in the mental component of ontogenesis. Each such developmental period is task-oriented. If in the sensory period of certain functions formation the society offers conditions not corresponding to the real preconditions of the child's development, the formation of functional systems of the brain may follow a disharmonious path of development. As a result, more intensive and advanced development of some functions can lead to increased differentiation of the brain and "stealing" in the development of those functions that are at the stage of their optimal formation. Otherwise, as a result of insufficient impact of external social factors, there is underdevelopment of higher mental functions and a decrease in the rate of their formation in relation to the average population level of development, which allows us to talk about partial deficit of mental processes.

According to the results of this study, the functions with a long period of formation – these are the functions of the brain Block III, and the functions provided mainly by the left hemispheric sections of the cerebrum – are subject to the greatest individual variability during preschool age in correlation with the material prosperity of the family.

The most intensive rate of development of arbitrary activity regulation functions and serial organisation of movements provided by the work of the cerebrum anterior, as well as functions provided by the left-hemispheric strategy of information processing (auditory-verbal functions and analytical components of visual perception mediated by speech) with relatively isolated lag of motor functions and right-hemispheric components of visual-spatial information processing is observed in preschoolers from families with high material prosperity. Evidently, a targeted intensive speech exposure leads to increased verbal loads upon the child, thereby stimulating the development of the speech areas of the brain. According to several data [31], the classificatory method associated mainly with the work of the left hemisphere reaches a proper level of development only by the end of adolescence, and the structural method, carried out by the right hemisphere, has a certain degree of maturity already in the period of preschool age. Early intensive verbal load can be considered to create prerequisites for the active development of left hemisphere functions and "steal" right hemispheric capabilities, including kinaesthetic functions and some components of visual-spatial information processing. Since visual and spatial functions are associated with the work of long-forming tertiary sections of the cortex of the brain Block II, including the right hemisphere, which reach their developmental optimum at an older age and are closely related to the learning process, it is essential to further investigate the dynamics of their development, which would enable us to clarify the distant consequences of a more intensive rate of formation of functions provided by the left hemisphere.

As family material prosperity decreases, the risk of "weakening" most of children's functional abilities increases. Children from low-income families are likely to have deficits in the functions of programming and control of arbitrary forms of activity, serial organisation of movements, mainly speech, as well as impaired ability to process auditory information and some speech-mediated analytical components of visual perception. Notwithstanding the fact that the decrease in material prosperity of the family is accompanied by a weakening of verbal functions and activity arbitrary regulation, it has a positive effect on the formation of kinesthetic hand praxis and some aspects of visual-spatial functions. Considering this variant of the child's norm, one may consider

that the decrease in the functional capabilities of the anterior and sinistrocerebral parts of the cerebrum reflects a variant of the partial deficit of the mental component of ontogenesis.

CONCLUSION

Every society is characterised by socio-economic inequalities, but the most effective social strategy that can improve the quality of life of the population in any country is to invest in child development: the earlier in childhood this contribution is made, the more effective the social returns will be years later.

Since the cerebrum in the early period is highly plastic and represents an open system capable of restructuring, the preschool age is such an important period for studying systemic regularities of correlations between individual variability of higher mental functions and factors of the family environment. It is in the preschool age that the variability in morphofunctional cerebral development reaches more than 30 %, and then there is a gradual decrease in the "plasticity" of the cerebrum, and the environmental influence begins to inevitably lose its relevance [32]. Thus, remedial interventions become less effective. Consequently, it is important that early experiences meet children's developmental potentials and that the differing socio-demographic characteristics of the family are taken into account in the design of the teaching and learning process.

The presented conceptual approaches, within the framework of which the search for connecting links between socio-demographic parameters of the family and structural and functional self-organisation of higher mental functions is conducted, were supplemented by empirical data of our study, which made it possible to specify that the factor of material wealth of the family both directly, factor-wise, and indirectly, cumulatively, through the system of proximal factors, can make its selective contribution to the variability of indicators of children's higher mental functions, which is manifested in more intensive formation of some groups of functions, and less intensive – others, as well as in the choice of the leading strategy of information processing, relying on the activity of the left or right hemisphere.

In conclusion, we note that the unevenness in the development of structural and functional components of children's higher mental functions is normative, not pathological. Such unevenness has a large adaptive effect: the population as a whole benefits from the presence of different abilities in different individuals. On the other hand, the transition to a new world order and a new technological mode, to new principles of social selection puts high demands on the psychophysiological characteristics of children. Under these conditions, the uneven development of higher mental functions can lead to the fact that weak structural links of the functional system can become a deterrent to further development and successful learning of the child. The results of this study can be useful to parents, teachers, specialists of preschool and other educational organizations. Analyzing the development of children before school and identifying their living conditions makes it possible to identify different groups of children who need help from the first days of school life.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the author

Marina S. Nerovnykh – Senior Lecturer at the Department of Psychological and Pedagogical Education, Katanov Khakass State University, e-mail: s.mariana@list.ru, https://orcid.org/0000-0001-5729-0019

ASSESSMENT OF CONSEQUENCES OF COVID-19 IN ADOLESCENTS BY THE METHOD OF QUESTIONNAIRE

Cherevikova I.A.,
Tkachuk E.A.,
Polyakov V.M.,
Vasileva N.S.,
Prokhorova Zh.V.,
Votineva A.S.,
Myasishchev N.A.

Scientific Centre for Family Health and Human Reproduction Problems (Timiryazeva str. 16, Irkutsk 664003, Russian Federation)

Corresponding author:
Irina A. Cherevikova,
e-mail: gothic.craze@mail.ru

ABSTRACT

Background. *In Russia, of all detected cases of COVID-19, 18 % were in the pediatric population. According to a number of studies, adolescents develop long-term clinical and psychological consequences after an illness. Therefore, at present, the most relevant is a thorough study of the structure and severity of consequences of COVID-19 in adolescents.*

The aim of the study. *To assess the severity of consequences of COVID-19 in adolescents depending on the time after the disease.*

Materials and methods. *The sample included 96 people aged 11–16 years: 48 adolescents who have undergone COVID-19 (main group); 48 adolescents who did not have COVID-19 (control group). The main group was divided into six subgroups, depending on the period after COVID-19. The following research methods were used: clinical history using a standardized interview method; "Correction test" method by B. Bourdon; Beck's Depression Inventory (BDI-1A); Adolescent's Form of Manifest Anxiety Scale by A.M. Prikhozhan.*

Results. *Clinical symptoms of COVID-19 during the acute phase of the disease were considered in adolescents. It has been established that the most common symptoms include fever, runny nose, cough, sore throat, severe fatigue, impaired sense of smell, impaired taste, headache (most often localized in the frontal region). Clinical and psychological symptoms characteristic of post-COVID syndrome were considered as consequences of COVID-19 in adolescents. The majority of the examined adolescents showed the following clinical symptoms of COVID-19 after discharge: asthenia, disturbances of smell and taste; lasting from 2 to 64 weeks. As psychological symptoms, adolescents were characterized by reduced attention span, reduced speed of information processing and concentration, as well as the presence of symptoms of severe depression and high anxiety. The most unfavorable emotional state was revealed in adolescents during the second month after COVID-19.*

Conclusion. *The data obtained made it possible to determine that post-COVID syndrome in adolescents is characterized by the presence of an asthenic condition, impaired attention, high anxiety, severe depressive symptoms.*

Key words: *COVID-19, long-COVID, post-COVID, adolescents*

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ОЦЕНКА ПОСЛЕДСТВИЙ COVID-19 У ПОДРОСТКОВ МЕТОДОМ АНКЕТИРОВАНИЯ

Черевикова И.А.,
Ткачук Е.А.,
Поляков В.М.,
Васильева Н.С.,
Прохорова Ж.В.,
Вотинева А.С.,
Мясищев Н.А.

ФГБНУ «Научный центр проблем здоровья, семьи и репродукции человека» (664003, г. Иркутск, ул. Тимирязева, 16, Россия)

Автор, ответственный за переписку:
Черевикова Ирина Александровна,
e-mail: gothic.craze@mail.ru

РЕЗЮМЕ

Обоснование. *В России из всех выявленных случаев COVID-19 18 % приходятся на население детского возраста. По данным ряда исследований, у подростков формируются долгосрочные клинические и психологические последствия после перенесённого заболевания. Следовательно, в настоящее время наиболее актуальным является тщательное изучение структуры и степени выраженности последствий COVID-19 у подростков.*

Цель исследования. *Оценить степень выраженности последствий COVID-19 у подростков в зависимости от временного периода после перенесённого заболевания.*

Методы. *Выборка включала 96 человек в возрасте 11–16 лет: 48 подростков, перенёсших COVID-19 (основная группа); 48 подростков, не болевших COVID-19 (контрольная группа). Основная группа разделена на 6 подгрупп в зависимости от временного периода после COVID-19. В качестве методов исследования использовались: клинический анамнез с использованием метода стандартизированного интервью; методика «Корректурная проба» Б. Бурдона; шкала депрессии А. Бека (BDI-1A); шкала явной тревожности для подростков А.М. Прихожан.*

Результаты. *Рассмотрены клинические симптомы COVID-19 во время острой фазы течения заболевания. Установлено, что к наиболее распространённым симптомам относятся: повышенная температура тела, насморк, кашель, боль в горле, сильная усталость, нарушения обоняния, нарушения вкуса, головная боль (наиболее часто локализованная в лобной области). В качестве последствий COVID-19 у подростков рассмотрены клинические и психологические симптомы, характерные для постковидного синдрома. У большинства обследованных подростков выявлены следующие клинические симптомы COVID-19 после выписки: астения, нарушения обоняния и вкуса продолжительностью от 2 до 64 недель. В качестве психологических симптомов, для подростков характерны: сниженный объём внимания, пониженный уровень скорости переработки информации и концентрации внимания, а также наличие симптомов выраженной депрессии и высокой тревожности. В период второго месяца после COVID-19 выявлено наиболее неблагоприятное эмоциональное состояние.*

Заключение. *Полученные данные показали, что для постковидного синдрома у подростков характерно наличие астенического состояния, нарушения внимания, высокой тревожности, симптомов выраженной депрессии.*

Ключевые слова: *COVID-19, продолженный COVID, постковид, подростки*

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INTRODUCTION

In Russia, cases of COVID-19 infection among the population have been recorded from 2020 onwards. Thus, according to official statistics, more than 18 million cases of COVID-19 infection have been detected in Russia since the beginning of the pandemic. At the same time, 18 % of diagnosed cases of COVID-19 infection are in the pediatric population, of which 6 % are pre-school children and 12 % are school-aged children. It is important to note that COVID-19 prevalence in the population is currently declining, which has allowed the removal of earlier restrictive measures (e. g., wearing masks). Nevertheless, at the same time, the effects of the disease in the population are still being felt. This is associated with the fact that COVID-19, in contrast to "classical" acute respiratory viral infections, is characterised by peculiarities of course and complications that can affect almost all human organs and systems [1–3]. In particular, the National Institute for Health and Care Excellence (NICE) has proposed a classification of COVID-19 course periods, according to which they differentiate: (1) acute period, when signs and symptoms of the disease persist for no more than 4 weeks (the diagnosis is removed when a negative PCR test for SARS-CoV-2 carriage is obtained); (2) long-COVID – persisting symptoms of the disease for 1–3 months; (3) post-COVID – when signs and symptoms that develop during and/or after the disease last for more than 3 months and are not explained by an alternative diagnosis [4]. These symptoms can change over time, disappear and reappear, affecting many body systems [5–7]. At the same time, it is noted that even those people who have experienced COVID-19 in a mild form are not immune to adverse effects [5, 8].

According to a number of studies, symptoms occurring during the acute period of COVID-19 course include fever, runny nose, cough, sore throat, gastrointestinal disorders, conjunctivitis, headache, severe fatigue, sleep disruption, smell and taste disorders [6, 9–11]. Various disorders of smell and taste were the most prominent symptoms of the acute period of COVID-19, and their prevalence reached 73–98 % [10–12]. The duration of these disorders, as a rule, is no more than 10–20 days, but cases of longer loss of sense of smell and taste – more than 60 days – are common [13], which are part of the structure of the so-called "long-COVID" and "post-COVID" [14].

A number of clinical and psychological symptoms characteristic of the long-COVID and post-COVID course of the disease are also distinguished. Currently, the symptomatology of these periods of the COVID-19 course is combined into a single nosology, post-COVID-19 syndrome. The diagnosis of post-COVID syndrome is listed in the International Classification of Diseases 10th Revision (ICD-10) under the code U09.9 "Condition after COVID-19 unspecified", which also includes post-COVID. In this case, post-COVID syndrome is defined as a complex of symptoms occurring during and/or after the disease, which persist for more than 3 months and are not explained by an alternative diagnosis [15, 16]. According to a study conducted in the Volgograd region, the prevalence of post-COVID syndrome among re-infected adolescents is 25 %, with symptoms persisting for 5 or more months [17]. That said, the various symptoms of post-COVID manifest with a different frequency and can vary. Thus, the most common clinical and psychological symptoms of post-COVID in adolescents include headache (3–80 %), abdominal pain (1–76 %), taste and smell disorder (3–74 %), sleep disruption (2–63 %), fatigue (3–87 %), depressive state (43–87 %), high anxiety (41–84 %), reduced attention concentration (2–81 %) [8, 18]. At the same time, however, the duration of symptoms has an individual severity. Thus, according to N.R. Magson et al. [19], the following symptoms are observed in adolescents during 4 to 12 weeks after the acute phase of COVID-19: headache, fatigue, sleep disruption, and difficulty concentrating. Different results from the previous study were obtained in a study by Q. Han et al. [20]. According to the authors, 8591 patients had symptoms such as fatigue/weakness (28 %), dyspnoea (18 %), arthromyalgia (26 %), depression (23 %), anxiety (22 %), memory disorder (19 %), decreased concentration (18 %) and insomnia (12 %) for at least 1 year [20].

Despite the significant contributions of these authors, the problem of identifying and studying the effects of COVID-19 in adolescents remains unresolved. Therefore, **the aim of this study** was to evaluate the severity of the COVID-19 consequences in adolescents according to the follow-up intervals.

MATERIALS AND METHODS

The study, conducted between November 2021 and June 2023, included adolescents from among the patients of the Clinic of the Scientific Centre for Family Health and Human Reproduction Problems (Irkutsk) who met the inclusion criteria. Inclusion criteria: age 11–16 years; informed consent of the adolescent's legal representative to participate in the study. Exclusion criteria: failure to meet the inclusion criteria; presence of comorbid disease (asthma, arterial hypertension, etc.); presence of disorder of mental development (mental retardation, autism spectrum disorder, cerebral palsy, etc.); refusal of the adolescent or his/her legal representative to participate in the study.

The pilot study was a prospective, non-randomized, case-control study. The study sample consisted of 96 adolescents: 18 (18.7 %) boys and 78 (81.3 %) girls aged 11–16 years (mean age – 14.6 ± 1.6 years). All the study participants were divided into two groups – main and control. Inclusion criteria in the main group: a history of a positive PCR test for SARS-CoV-2 carriage and evidence of mild or moderate COVID-19. Inclusion criteria for the control group: no history of a positive PCR test for SARS-CoV-2 carriage and no evidence of mild or moderate COVID-19. In order to assess the severity of the COVID-19 consequences in adolescents according to the follow-up interval, the main group was divided into six subgroups: (1) one month after COVID-19; (2) two months after COVID-19; (3) three months after COVID-19; (4) four months after COVID-19; (5) six

months to one year after COVID-19; (6) more than one year after COVID-19. Table 1 summarizes the general characteristics of the study groups of adolescents. The main and control groups of the study were comparable in sex and age characteristics.

All respondents were surveyed using the following methods: clinical history using the standardised interview method; B. Bourdon's Test [21]; Beck Depression Inventory (BDI-1A) adapted by N.V. Tarabrina [22]; A.M. Prikhozhan's manifest anxiety scale for adolescents [23].

Clinical history using standardised interview method included collection of anamnestic information about respondents (sex, age, presence of COVID-19 cases in the family, features of the course of the acute phase of COVID-19, features of the condition after COVID-19 diagnosis removal, presence of stressful experiences at the time of examination).

To assess the properties of respondents' attention, the method of B. Bourdon's Test was used [21]. The survey was conducted using special forms with rows of letters arranged in random order. Each respondent viewed a series of letters and crossed out certain letters indicated in the instructions for 7 minutes. At the end of each minute, the respondent made a mark where he/she held the pencil/pen. The following indicators were determined based on the results of the method: attention span, attention concentration, accuracy and speed.

The "attention span" indicator corresponded to the total number of letters viewed by the respondent in 7 minutes.

To determine the "concentration of attention" indicator (P), the "concentration of attention in 1 minute" indicator was first calculated for each of the 7 minutes according to the formula:

$$P = K \times Q,$$

where: P – attention concentration in 1 minute; K – accuracy in 1 minute; Q – number of letters viewed in 1 minute.

Then the "concentration of attention" indicator (Pt) was calculated using the formula:

$$Pt = (P1 + P2 + P3 + P4 + P5 + P6 + P7) / 7,$$

where: $P(1–7)$ is the concentration for each individual minute.

To determine the "accuracy" indicator, the "accuracy in 1 minute" indicator (K) was first calculated for each of the 7 minutes according to the formula:

$$K = (n – x) / n,$$

where: n – number of letters to cross out in 1 minute; x – number of errors in 1 minute.

Then the "accuracy" indicator (Kp) was calculated using the formula:

$$Kp = (K1 + K2 + K3 + K4 + K5 + K6 + K7) / 7,$$

where: $K(1–7)$ is the accuracy for each individual minute.

To determine the "speed" indicator (S), the formula was used:

$$S = (0{,}5936 \times Qt – 2{,}807 \times xn) / t,$$

where: Qt is the total number of letters viewed in 7 minutes; xn – the total number of errors in 7 minutes; 0.5936 – the average volume per letter; 2.807 – the loss of information per missed letter; t – the total time of the technique (in seconds).

To assess the presence of symptoms of depression in adolescents, the Beck Depression Scale (BDI-1A) was used, developed by A. Beck in 1978 and adapted by N.V. Tarabrina in 2001 [22]. The scale contains 13 groups of statements corresponding to groups of depression symptoms. Each item on the scale is rated from 0 to 3 points according to increasing severity of symptoms. Thus, the final result of the method from 0 to 9 points indicates the absence of symptoms of depression; from 10 to 15 points – the presence of symptoms of mild depression (subdepression); from 16 to 19 points – the presence of symptoms of moderate depression; more than 20 points – the presence of symptoms of severe depression [23].

A.M. Prikhozhan's manifest anxiety scale for adolescents [24] was used to identify anxiety as a relatively sta-

TABLE 1

GENERAL CHARACTERISTICS OF THE STUDIED GROUPS OF ADOLESCENTS

Groups	Age (years), M ± SD	Sex, % (*n*)		Total, % (*n*)
		boys	girls	
Main group	14.6 ± 1.6	18.7 (*n* = 9)	81.3 (*n* = 39)	100.0 (*n* = 48)
Subgroup 1	14.7 ± 0.1	14.3 (*n* = 1)	85.7 (*n* = 6)	14.6 (*n* = 7)
Subgroup 2	15.2 ± 0.6	20.0 (*n* = 1)	80.0 (*n* = 4)	10.4 (*n* = 5)
Subgroup 3	14.7 ± 0.3	14.3 (*n* = 1)	85.7 (*n* = 6)	14.6 (*n* = 7)
Subgroup 4	14.4 ± 0.1	37.5 (*n* = 3)	62.5 (*n* = 5)	16.7 (*n* = 8)
Subgroup 5	14.5 ± 0.3	8.4 (*n* = 1)	91.6 (*n* = 11)	25.0 (*n* = 12)
Subgroup 6	14.4 ± 0.4	22.2 (*n* = 2)	77.8 (*n* = 7)	18.7 (*n* = 9)
Control group	14.6 ± 1.6	18.7 (*n* = 9)	81.3 (*n* = 39)	100.0 (*n* = 48)
Total	14.6 ± 1.6	18.7 (*n* = 18)	81.3 (*n* = 78)	100.0 (*n* = 96)

ble personality formation in adolescents. The scale developed by A.M. Prikhozhan on the basis of adult and child versions of the manifest anxiety scale (J. Taylor, 1951, 1953; A. Castenada, B.R. McCandless, D.S. Palermo, 1956) contains 65 items. Analyzing the respondent's answers allows us to calculate "raw" scores on the anxiety scale. "Raw" scores are then converted to scale scores (walls) by comparing the examinee's data with normative scores of a group of adolescents of the appropriate age and gender. Based on the obtained scale score, the level of anxiety expression of the respondent was determined. Thus, 1–2 walls indicate the presence of a low level of anxiety; 3–6 walls indicate a normal level of anxiety; 7–8 walls indicate somewhat elevated anxiety; 9 walls indicate high anxiety; 10 walls indicate very high anxiety.

The study protocol was designed in accordance with the World Medical Association Declaration of Helsinki "Ethical Principles for the Conduct of Scientific Medical Research Involving Human Subjects" as amended in 2013.

Statistical processing of the study results was performed using Statistica 8 application software package (StatSoft Inc., USA). The sample size has not been precalculated. The Shapiro – Wilk test was used to check the normality of the distribution of the studied indicators. Arithmetic mean and standard deviation in M ± SD format were used to describe quantitative data. The Mann – Whitney U-criterion was used to analyze intergroup differences. Differences in percentage or relative values were assessed using Pearson's χ^2 criterion. The critical value of the level of statistical significance was considered to be $p \leq 0.05$.

RESULTS

The frequency and duration of clinical symptoms of COVID-19 in adolescents during the course of the acute period of the disease were determined based on the re-

TABLE 2

FREQUENCY AND DURATION OF CLINICAL SYMPTOMS OF COVID-19 IN ADOLESCENTS

Clinical symptoms	Detection frequency, *n* (%)	Duration from disease onset (days), M ± SD	Duration (day), min–max
Elevated body temperature	41 (85 %)	8.7 ± 4.7	1–21
Severe fatigue	34 (71 %)	19 ± 6.9	7–38
Runny nose	32 (67 %)	6 ± 4.3	1–21
Olfactory disorders	28 (58 %)	19 ± 6.9	7–38
Headaches, of which:	29 (60 %)		
temporal region	3 (10 %)		
occipital region	3 (10 %)		
frontal region	12 (42 %)	19 ± 6.9	7–38
frontal and parietal region	2 (7 %)		
frontal and temporal region	2 (7 %)		
whole head	7 (24 %)		
Cough	27 (56 %)	6 ± 4.3	1–21
Abnormal taste	26 (54 %)	19 ± 6.9	7–38
Sore throat	27 (56 %)	6 ± 4.3	1–21
Sleep disruptions:	14 (29 %)		
hard to sleep	10 (21 %)		
nocturnal awakening	7 (15 %)	19 ± 6.9	7–38
nightmares	3 (6 %)		
hard to wake up	6 (13 %)		
Gastrointestinal disorders	7 (15 %)	6 ± 4.3	1–21
Conjunctivitis	4 (8 %)	6 ± 4.3	1–21
Total period of illness	48 (100 %)	17 ± 8.3	1–38

sults of the clinical history of the disease (Table 2). Specifically, the acute period of the course of COVID-19 in adolescents averaged 17 ± 8.3 days (1 to 38 days).

Table 2 summarizes that more than half of the respondents reported the following clinical symptoms: fever (85 %), runny nose (67 %), cough (56 %), sore throat (56 %), severe fatigue (71 %), olfactory disorder (58 %), taste disorder (54 %), headache (60 %) (most often localised in the frontal area). The following COVID-19 clinical symptoms were less frequent: gastrointestinal disorders (15 %), conjunctivitis (8 %), and sleep disruptions (29 %) (most commonly associated with difficulty falling asleep).

Based on the results of the clinical history of the disease, the frequency and duration of COVID-19 clinical symptoms in adolescents after the diagnosis was removed (during the course of the post-COVID syndrome) were determined (Table 3). Specifically, 77 % of adolescents showed COVID-19 clinical symptomatology after the diagnosis was removed for a duration ranging from 0.5 to 64 weeks (mean – 10.1 ± 14.6 weeks).

The most common clinical symptoms of COVID-19 after the diagnosis has been removed are: asthenia, smell and taste disorders, as can be summarised in Table 3. Symptoms such as headache, joint pain, runny nose, cough, ele-

TABLE 3

FREQUENCY AND DURATION OF COVID-19 CLINICAL SYMPTOMS IN ADOLESCENTS AFTER THE DIAGNOSIS HAS BEEN REMOVED

Clinical symptoms	Detection frequency, *n* (%)	Duration after discharge (weeks), M ± SD	Duration (weeks), min–max
Joint ache	2 (4 %)	1.8 ± 0.4	1.5–2.0
Runny nose	1 (2 %)	0.5 ± 0.0	0.5–0.5
Elevated body temperature (37 °C)	3 (6 %)	13.8 ± 13.3	1.5–28.0
Cough	1 (2 %)	1.0 ± 0.0	1.0–1.0
Diarrhea	1 (2 %)	1.0 ± 0.0	1.0–1.0
Severe chest pain	1 (2 %)	1.0 ± 0.0	1.0–1.0
Reduced appetite	2 (4 %)	26.0 ± 2.8	24.0–28.0
Nausea	2 (4 %)	2.5 ± 1.3	1.0–4.0
Dizziness	3 (6 %)	5.0 ± 4.6	2.0–12.0
Headache	2 (4 %)	5.0 ± 4.8	1.0–12.0
Asthenia	15 (31 %)	14.0 ± 12.1	3.0–64.0
Abnormal taste	16 (33 %)	11.7 ± 10.5	2.0–56.0
Olfactory disorders	17 (36 %)	14.6 ± 10.3	2.0–64.0
General post-COVID period	37 (77 %)	10.1 ± 14.6	0.5–64.0

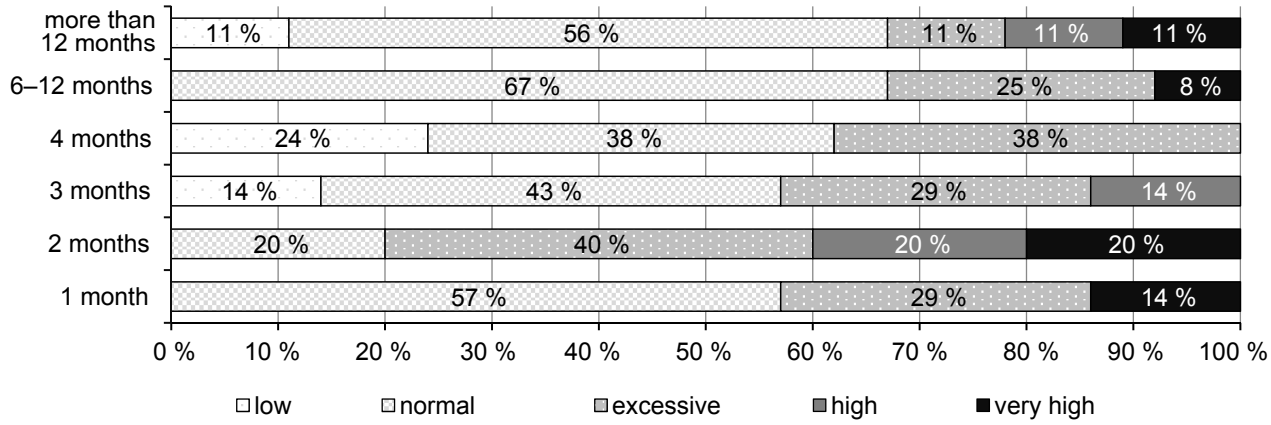

FIG. 1.

Anxiety levels in adolescents at different periods after COVID-19

vated body temperature, dizziness, nausea, diarrhoea, severe chest pain, and decreased appetite, were however rare and ranged from 0.5 to 28 weeks after COVID-19.

An analysis of adolescents' anxiety severity as a function of time after COVID-19 is summarised in Figure 1.

High anxiety (high and very high levels) was observed in adolescents between 1 and 3 months and six months and more than 12 months after COVID-19, as can be found in Figure 1. The majority of adolescents were characterised by high anxiety in the second month after the disease. Along with this, the detected differences in the values of respondents' anxiety severity indices in correlation with the time period after COVID-19 were not statistically significant ($p > 0.05$; χ^2 Pearson).

An analysis of depression symptom severity in adolescents after COVID-19 is summarised in Figure 2.

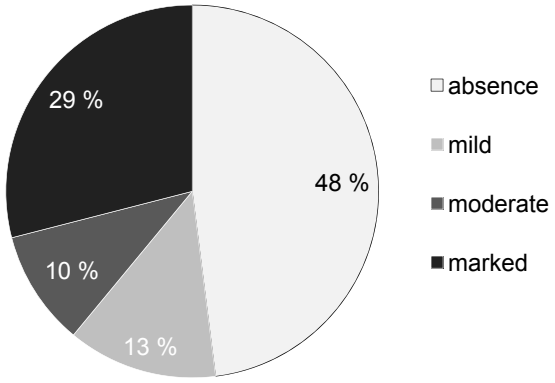

FIG. 2.
The severity of depressive symptoms in adolescents after COVID-19

Figure 2 indicates that the majority of adolescents (52 %) were diagnosed with symptoms of depression after undergoing COVID-19. For instance, 29 % of surveyed adolescents were diagnosed with symptoms of marked depression, 10 % with symptoms of moderate depression, and 13 % with symptoms of mild depression.

The analysis of depression symptom severity in adolescents as a function of time after COVID-19 is summarized in Figure 3.

Figure 3 reveals that the majority of adolescents (60 %) had symptoms of severe depression at 2 months after COVID-19. Symptoms of moderate depression were diagnosed in 29 % of adolescents in the first month after COVID-19; in 12 % of adolescents in 4 months after COVID-19; in 8 % of adolescents in 6 months to 1 year after COVID-19; and in 11 % of adolescents in 1 year or more after COVID-19. Mild depression symptoms were diagnosed in 14 % of adolescents at 1 month after COVID-19; in 40 % of adolescents at 2 months after COVID-19; in 12 % of adolescents at 4 months after COVID-19; and in 17 % of adolescents at 6 months to 1 year after COVID-19. The differences found are not statistically significant ($p > 0.05$; Pearson's χ^2).

In order to reveal the features of the COVID-19 consequences in adolescents, the properties of attention, the presence of symptoms of depression and the level of anxiety in adolescents of the main and control groups were analysed. Characteristics of attention properties in adolescents of the main and control groups are summarised in Table 4.

Table 4 reveals that adolescents who had undergone COVID-19 (main group) showed reduced attention span, reduced information processing speed and concentration. Statistically significant differences were revealed between the respondents of both groups based on the level of attention span, attention concentration and speed of information processing ($p \leq 0.05$).

Figure 4 illustrates the mean values of depression and anxiety symptom severity in adolescents of the main and control groups.

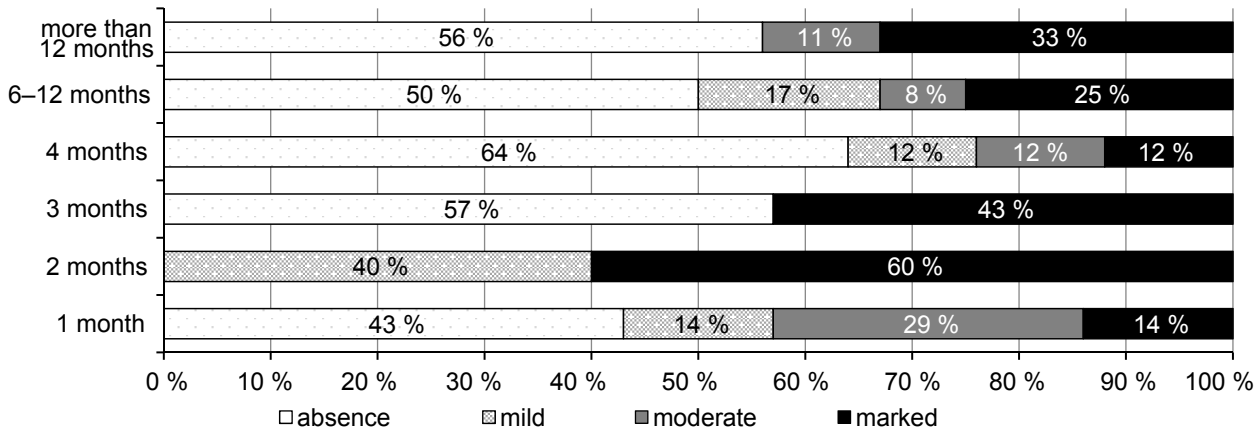

FIG. 3.
Depression symptom severity in adolescents at different periods after COVID-19

TABLE 4

ATTENTION FEATURES OF ADOLESCENTS IN THE MAIN AND CONTROL GROUPS

Indicator	Main group, M ± SD	Control group, M ± SD	Statistical significance level, *p*
Attention span	1060.40 ± 240.27	1311.90 ± 354.73	0.026*
Concentration (attentiveness)	138.28 ± 29.73	164.90 ± 41.84	0.042*
Accuracy	0.92 ± 0.07	0.87 ± 0.08	0.117
Speed/velocity	1.34 ± 0.29	1.63 ± 0.43	0.038*

Note. * – differences are statistically significant by Mann – Whitney U-criterion.

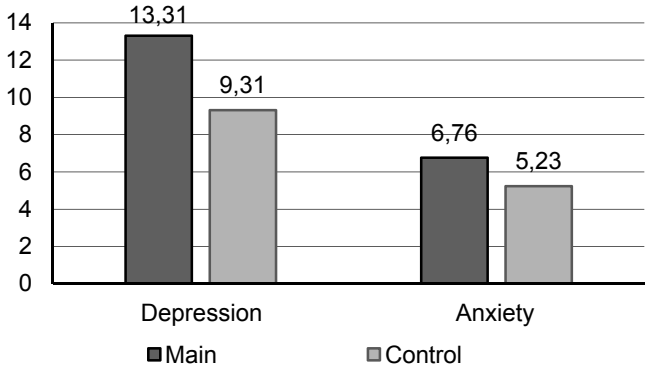

FIG. 4.

Depression and anxiety symptoms in adolescents of the main and control groups

In assessing the depression and anxiety symptom severity, higher levels of depression ($p = 0.002$) and anxiety ($p = 0.033$) were revealed in the group of respondents who had undergone COVID-19 (Fig. 4).

DISCUSSION

The results obtained in this study allow the authors to conclude that the clinical symptoms of COVID-19 characteristic of adolescents during the acute period of the disease include the following: fever (85 %), runny nose (67 %), cough (56 %), sore throat (56 %), severe fatigue (71 %), olfactory disorder (58 %), taste disorder (54 %), headache (60 %) (most often localised in the frontal area). These findings correlate with the results of a study by A. Nalbandian et al. who found a similar incidence of COVID-19 clinical symptomatology in adolescents during the course of the acute period of the disease [9].

Compared to the data of a study conducted in the Volgograd region [17], the prevalence and duration of clinical symptoms of COVID-19 after the diagnosis was removed were found to be higher. The incidence of these symptoms among overexposed adolescents, according to the results of our study, is 77 %, and their duration varies from 2 to 64 weeks. It is our hypothesis that this may be associated, firstly, with regional peculiarities of PCR testing coverage for SARS-CoV-2 carriage; secondly, with the fact that this study did not retrospectively analyze data from patients' ambulatory records and medical histories, but used information about clinical symptoms COVID-19 during the illness and after discharge, provided by the respondents themselves during the oral interview.

It has been revealed that the most common clinical symptoms of COVID-19 after discharge include: asthenia (31 %), olfactory disorder (36 %) and abnormal taste (33 %). This is supported by the research data of K.I. Usov [8] and A.M. Bogariu et al. [18], according to the data of which, in adolescents in the post-COVID period are observed: from 3 to 87 % – fatigue; from 3 to 74 % – disorder of taste and smell. Meanwhile, according to the results of this study, the majority of respondents noted distortion of the taste to meat and dairy products ("rotten", "unpalatable"), which led to their complete exclusion from the diet until the relief of the symptom.

Analyses of anxiety and depression symptoms in adolescents based on the time period after COVID-19 revealed the following trends: 1) at least 15 % of the studied adolescents showed high anxiety in the periods from 1 to 3 months and from 1 year and more after COVID-19; 2) the majority of the studied adolescents (52 %) showed symptoms of depression after COVID-19; 3) symptoms of severe depression were revealed in adolescents in all the studied periods after COVID-19; 4) the most unfavourable emotional state in adolescents was revealed in the period of the second month after COVID-19. The statistical analysis of the detected differences in the values of anxiety and depression symptoms of the respondents of these groups did not show statistically significant differences. The reason for this may be that COVID-19 disease in adolescents may cause changes in the emotional sphere at a deeper level, affecting issues of self-image, perception of the world around and general stress tolerance, which leads to the appearance of symptoms of severe depression regardless of the time period after the disease.

The comparative analysis of attention properties, presence of depression symptoms and anxiety level in adolescents of the main and control groups allowed to reveal the peculiarities of the course of post-COVID syndrome

in adolescents. For instance, in the group of adolescents who underwent COVID-19, compared to the respondents of the control group, the following were revealed:

1. Reduced attention span, reduced level of information processing speed and attention concentration, which may be associated with the presence of a state of increased vigilance and tension in respondents who have undergone COVID-19.

2. Presence of symptoms of severe depression and high levels of anxiety. These conditions may be associated with the neurotropism of the SARS-CoV-2 virus; the social significance of the disease itself; social influence (news, being in quarantine and isolation, etc.); the emergence of feelings of guilt, feelings of failure and despair, negative attitudes towards oneself (in the case of infected relatives, older generation).

Study limitations

Despite the obtained statistically significant differences in the level of anxiety, depression symptoms and attention properties in adolescents depending on the presence or absence of a COVID-19 history, this study was a pilot study in this age group and has a number of limitations. These include the small sample size and the predominance of female adolescents in the sample. Also, this study utilized information about existing COVID-19 clinical symptoms during the illness and after discharge provided by the respondents themselves in an oral interview. This could have been one source of inaccuracy in the study. The presence of these limiting factors constrains the extension of the findings to the general population of adolescents and requires additional research.

CONCLUSION

The results of this study enabled assessment of the severity of the COVID-19 consequences in adolescents depending on the period after the disease. Clinical symptoms of COVID-19 in adolescents during the acute phase of the disease course are considered. It has been established that the most common symptoms include fever, runny nose, cough, sore throat, severe fatigue, impaired sense of smell, impaired taste, headache (most often localized in the frontal region).

It has been revealed that 77 % of adolescent post-COVID-19 cases exhibit post-COVID symptomatology lasting between 2 and 64 weeks. Meanwhile, the most common clinical symptoms of COVID-19 after discharge include: asthenia (31 %), olfactory disorder (36 %) and abnormal taste (33 %). The most common psychological symptoms characteristic of post-COVID syndrome include: reduced attention span, reduced information processing speed and concentration, as well as the presence of symptoms of marked depression and high anxiety. In summary, it can be said that the second month post COVID-19 revealed the most unfavourable emotional state in adolescents. The severity of depression and anxiety symptoms did not statistically significantly change depending on the time after COVID-19. Therefore, the next stage of this ongoing study is to increase the sample size in order to observe the socio-demographic, psychological and physiological determinants of health-related quality of life in children who have undergone COVID-19.

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Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Irina A. Cherevikova – Junior Research Officer at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: gothic.craze@mail.ru, https://orcid.org/0000-0002-5328-8525

Elena A. Tkachuk – Dr. Sc. (Med.), Senior Research Officer at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: zdorowie38@gmail.ru, https://orcid.org/0000-0001-7525-2657

Vladimir M. Polyakov – Dr. Sc. (Biol.), Leading Research Officer at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: vmpolyakov@mail.ru, https://orcid.org/0000-0001-6243-9391

Nadezhda S. Vasileva – Clinical Research Assistant at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: wasns-irk@mail.ru, https://orcid.org/0000-0002-2648-3528

Zhanna V. Prokhorova – Cand. Sc. (Med.), Research Officer at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: proxorowa.janna2011@yandex.ru, https://orcid.org/0000-0001-8236-1747

Anastasiya S. Votineva – Junior Research Officer at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: votinevaas@gmail.com, https://orcid.org/0000-0002-0361-2868

Nikolai A. Myasishchev – Clinical Research Assistant at the Laboratory of Pediatric Psychoneurosomatic Pathology, Scientific Centre for Family Health and Human Reproduction Problems, e-mail: roulih@mail.ru, https://orcid.org/0000-0001-8451-9341

PHARMACOLOGY AND PHARMACY

THE EFFECT OF COMPLEX PHARMACOTHERAPY REGIMENS USING A HERBAL REMEDY FROM *HIPPOPHAE RHAMNOIDES* ON BIOCHEMICAL BLOOD PARAMETERS OF RATS WITH PARACETAMOL HEPATITIS

Batueva S.Yu. [1],
Lamazhapova G.P. [1, 2],
Kapustina Yu.A. [1],
Rodnaeva O.A. [1],
Chukaev S.A. [1]

[1] D. Banzarov Buryat State University (Smolina str. 24A, Ulan-Ude 670000, Russian Federation)
[2] East Siberian State University of Technology and Management (Klyuchevskaya str. 40B, Ulan-Ude 670013, Russian Federation)

Corresponding author:
Snezhana Yu. Batueva,
e-mail: bsu.2017@mail.ru

ABSTRACT

Background. *The use of complexes of synthetic and herbal remedies as hepatoprotectors in the treatment of liver pathologies of various etiologies is an urgent task of pharmacology. A promising type of medicinal plant raw material is an extract of Hippophaes rhamnoides leaves. The hepatoprotective effect of extract of Hippophaes rhamnoides leaves in combination with ademethionine has not been studied to date.*

The aim of the work. *To study changes in biochemical markers of hepatic function in the application of complex schemes of pharmacotherapy of experimental liver damage with paracetamol using Extractum foliorum Hippophaes rhamnoides.*

Materials and methods. *The experimental study was performed on Wistar rats. The animals were divided into six groups. Group 1 (intact) – animals without a model of liver damage and without treatment; in Group 2 (control) paracetamol was used to create experimental hepatitis without treatment (positive control group); in Group 3 (comparison) a combination of "silibinin + ademethionine" was used on a model of paracetamol hepatitis; in Group 4 (experimental) extract of Hippophaes rhamnoides leaves was used on the model of paracetamol hepatitis; in Group 5 (experimental) ademethionine was used on a model of paracetamol hepatitis; in Group 6 (experimental) a combination of extract of Hippophaes rhamnoides leaves and ademethionine was used on a model of paracetamol hepatitis. The functional state of the liver of experimental animals was determined by biochemical parameters.*

Results. *When using a combination of extract of Hippophaes rhamnoides leaves with ademethionine, the studied biochemical parameters significantly ($p < 0.05$) differed from the numerical values in the negative control group and were closest to those in the intact group than in other experimental groups.*

Conclusion. *A comparison of the effectiveness of the use of extract of Hippophaes rhamnoides leaves in combination with ademethionine by the total effect on blood biochemical parameters determines this complex as a promising drug for further research.*

Key words: *Extractum foliorum Hippophaes rhamnoides, paracetamol experimental hepatitis, complex pharmacotherapy regimens, paracetamol, ademethionine, blood biochemical parameters*

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ВЛИЯНИЕ КОМПЛЕКСНЫХ СХЕМ ФАРМАКОТЕРАПИИ С ИСПОЛЬЗОВАНИЕМ ФИТОСРЕДСТВА ИЗ *HIPPOPHAE RHAMNOIDES* НА БИОХИМИЧЕСКИЕ ПОКАЗАТЕЛИ КРОВИ КРЫС С ПАРАЦЕТАМОЛОВЫМ ГЕПАТИТОМ

Батуева С.Ю. [1],
Ламажапова Г.П. [1, 2],
Капустина Ю.А. [1],
Роднаева О.А. [1],
Чукаев С.А. [1]

[1] ФГБОУ ВО «Бурятский государственный университет им. Д. Банзарова» (670000, г. Улан-Удэ, ул. Смолина, 24а, Россия)
[2] ФГБОУ ВО «Восточно-Сибирский государственный университет технологий и управления» (670013, г. Улан-Удэ, ул. Ключевская, 40в, Россия)

Автор, ответственный за переписку:
Батуева Снежана Юрьевна,
e-mail: bsu.2017@mail.ru

РЕЗЮМЕ

Обоснование. *Использование комплексов синтетических и растительных средств в качестве гепатопротекторов при лечении патологий печени различной этиологии является актуальной задачей фармакологии. Перспективным видом лекарственного растительного сырья является фитопрепарат – экстракт листьев облепихи крушиновидной (Extractum foliorum Hippophaes rhamnoides). Гепатопротекторное действие экстракта листьев облепихи крушиновидной в сочетании с адеметионином к настоящему времени не изучено.*

Цель работы*. Исследование изменений биохимических маркеров печёночной функции при применении комплексных схем фармакотерапии экспериментального повреждения печени парацетамолом с использованием Extractum foliorum Hippophaes rhamnoides.*

Методы*. Экспериментальное исследование выполнено на крысах линии Wistar. Животные были разделены на шесть групп: 1-я интактная группа – животные без модели повреждения печени и без лечения; 2-я контрольная группа – с применением парацетамола для создания экспериментального гепатита без лечения (группа негативного контроля); 3-я сравнительная группа – использование комбинации «силибинин + адеметионин» на модели парацетамолового гепатита; 4-я опытная группа – использование «экстракта листьев облепихи крушиновидной» на модели парацетамолового гепатита; 5-я опытная группа – использование «адеметионина» на модели парацетамолового гепатита; 6-я опытная группа – использование комбинации «экстракт листьев облепихи крушиновидной + адеметионин» на модели парацетамолового гепатита. Функциональное состояние печени экспериментальных животных определено по биохимическим показателям.*

Результаты. *При определении в сыворотке крови крыс уровня таких показателей, как аспарагиновая аминотрансфераза, аланиновая амионтрансфераза, щелочная фосфатаза, гамма-глютамилтранспептидаза, а также общего билирубина было установлено, что при применении сочетания экстракта листьев облепихи крушиновидной с адеметионином биохимические показатели статистически значимо ($p < 0,05$) отличались от численных значений в группе негативного контроля и были наиболее приближены к таковым в интактной группе, чем в других опытных группах.*

Заключение*. Сравнение эффективности применения экстракта листьев облепихи крушиновидной в сочетании с адеметионином по суммарному влиянию на биохимические показатели крови определяет данный комплекс как перспективную схему терапии парацетамолового гепатита.*

Ключевые слова: *Extractum foliorum Hippophaes rhamnoides, парацетамоловый экспериментальный гепатит, комплексные схемы фармакотерапии, парацетамол, адеметионин, биохимические показатели крови*

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BACKGROUND

Drug-induced liver injury is one of the important problems of modern hepatology. Many drugs are toxic to the body, even in therapeutic doses, which often leads to acute liver failure [1, 2]. A rather diverse arsenal of drugs that act differently against the liver cells is used for the therapy of various liver diseases [3]. Plant-derived drugs have a number of advantages over their synthetic counterparts in their effects on the homeostasis system. The bioactive metabolites produced by plants appear to be of interest as platforms for the synthesis of combinatorial combinations of synthetic and herbal drugs [4]. The use of various combinations of hepatoprotectors in the treatment of liver pathologies is quite in demand in modern conditions [5]. Pharmacological activity of *Hippophae rhamnoides foliae extract* is caused by the presence of quercetin, kaempferol, isoramnetin, catechins, flavone glycosides, etc. in the composition of the phytoextract [6, 7]. The evidence from various literature sources indicates that the presence of biologically active substances belonging to the group of phenols or polyphenols in pharmacological agents of natural origin defines their antioxidant properties [8–10]. Meanwhile, it should be emphasised that in general the spectrum of pharmacological activity of *Hippophae rhamnoides foliae extract*, peculiarities of action in conditions of combined use with other pharmacological agents have not been sufficiently studied to date.

THE AIM OF THE STUDY

To study changes in biochemical markers of hepatic function when applying complex schemes of pharmacotherapy of experimental liver injury with paracetamol using *Hippophae rhamnoides foliae* extract.

MATERIALS AND METHODS

The experimental study was performed using 60 white Wistar rats of both sexes weighing 180–200 g. The animals were kept in the certified vivarium of General and Experimental Biology Institute (GEBI) of the SB RAS (the Siberian Branch of Russian Academy of Sciences). All experiments were performed in accordance with the international rules for working with experimental animals, GOST 33044-2014, Principles of Good Laboratory Practice (OECD Guide 1:1998, IDT), according to the Order of the Government of the Russian Federation dated November 8, 2013 No. 2067-r. Animals were kept with free access to food and water on a diet that complied with GOST standards. The protocol of the experiment was approved by the Ethical Committee of the GEBI SB RAS (Minutes No. 2 dated November 5, 2017).

Before commencing the experiments, rats fulfilling the inclusion criteria for the study were allocated into six groups:

I. Intact rats.

II. Control group – animals with acute paracetamol-induced hepatitis.

III. Experimental group – combination "silibinin + ademethionine".

IV. Experimental group – phytopreparation "*Hippophae rhamnoides foliae* extract".

V. Experimental group – pharmacopoeial preparation "ademethionine" [10, 11].

VI. Experimental group – combination "*Hippophae rhamnoides foliae* extract + ademethionine".

Exclusion criteria for laboratory animals:

1) Presence or development of other somatic pathologies;

2) Immature, early age (up to 6 months);

3) With insufficient mass.

The experimental model of hepatitis in rats was reproduced by intragastric administration of paracetamol (acetaminophen) to animals once a day at a dose of 500 mg/kg for 2 days [12].

To treat experimental hepatitis, animals of experimental groups were orally administered daily in the morning hours (before feeding) for 14 days with aqueous solutions of the tested agents: *Hippophae rhamnoides foliae* phytoextract was dissolved in purified water at a dose of 100 mg/kg of animal body weight ("the most optimal pharmacotherapeutic" mode of administration [13]). *Hippophae rhamnoides foliae* extract was obtained by triple extraction with 40 % ethanol in GEBI SB RA; "silibinin" ("Vifitech") was administered at a dose of 200 mg/kg according to the scheme; "ademethionine" ("Veropharm") was administered at a dose of 200 mg/kg.

Distilled water in the first and second groups (intact animals with normofunction of the liver and with experimental hepatitis) was provided in equi-volume quantities according to the scheme.

Animals were removed from the experiment by immediate decapitation under light ether anesthesia.

At the moment of animal discharge from the experiment, blood was collected from the tail vein and the serum content was determined of:

1) asparagine aminotransferase (AST) (BioSystems, Spain; cat. no. 11830);

2) alanine aminotransferase (ALT) (BioSystems, Spain; cat. no. 11832);

3) alkaline phosphatase (ALP) (BioSystems, Spain; cat. no. 11832);

4) total bilirubin (BioSystems, Spain; cat. no. 11832);

5) gamma-glutamyl transpeptidase (GGTP) (BioSystems, Spain; cat. no. 11832).

Enzyme studies were performed by the kinetic method recommended by the German Society for Clinical Chemistry and the Clinical Manual for Laboratory Tests at 25 °C.

An automatic biochemical analyzer "Sapphire-400" (TokyoBoeki, Japan) and Human test systems (Germany) were used as a measuring instrument. Statistical data processing was performed using Statistica 6.0 for Windows program (StatSoft Inc., USA). Statistical significance of the differences between the compared values was calculated using the parametric Student's criterion and the non-

parametric Mann – Whitney U-criterion. Data differences were considered statistically significant at $p \leq 0.05$ [14].

RESULTS

According to the results of this study, it was found that AST level was statistically significantly increased in the group of animals with acute paracetamol hepatitis by 49.4 % ($p < 0.05$) compared to that in the intact group (Table 1).

The AST index in rats of the experimental groups did not statistically significantly differ from the observed values in the control group. However, the use of *Hippophae rhamnoides foliae* extract in combination with ademetionine contributed to a more significant decrease in AST value in comparison with other studied groups.

Under conditions of simulated experimental hepatitis, ALT level in the negative control group was statistically significantly increased by 22 % as compared to that in the intact group during the development of paracetamol-induced lesions than in the intact group, evidenced by the development of liver pathology. When comparing the studied index in group III with the same value in the control group in paracetamol hepatitis, it was found that ALT level decreased by 11.2 %, in Group IV – by 15.4 %, in Group V – by 11.1 %, in Group VI – by 12.4 %. However, statistically significant differences were registered only in Groups III and VI. In comparing the index of this aminotransferase in Group VI rats with paracetamol-induces hepatitis that received *Hippophae rhamnoides foliae* extract in combination with ademetionine and Group III rats that received silibinin with ademetionine, it can be seen that the ALT level was found to be identical. At the same time in animals of VI experimental group the smallest differences in ALT value were registered in comparison with that in intact animals.

The studies revealed that in animals with experimental paracetamol hepatitis the activity of alkaline phosphate and GGTP in serum was statistically significantly increased compared to the value in intact rats by 37.7 % and 75.7 %, respectively, as is evident from Table 1. The mean levels of alkaline phosphate and GGTP, which are marker enzymes of cholestasis, in the experimental groups were statistically significantly lower than in the control group of animals. In these conditions, the administration of *Hippophae rhamnoides foliae* extract in combination with ademetionine caused a statistically significant decrease in the level of alkaline phosphate activity by 13.2 % compared to the control. Similar changes were observed in the action of silibinin with ademetionine, *Hippophae rhamnoides foliae* extract and ademetionine separately: alkaline phosphate activity decreased by 12.9 %, 10.8 % and 12.1 %, respectively, relative to the control. GGTP enzyme level data in different experimental groups did not differ significantly from each other, and all of them were statistically significantly different from this index in rats of the control group.

After paracetamol administration, the bilirubin level in the control group increased by 114.3 % (Fig. 1).

In comparing the index of bilirubin in rats of the experimental group, administered silibinin with ademetionine, with that in animals of the control group, it was found that the level of bilirubin decreased by 31.1 %. With the values in Group IV this difference amounted to 24.4 %, in Group V – 33.3 %, in Group VI – 37.7 %. Using a combination of *Hippophae rhamnoides foliae* extract with ademetionine in rats with paracetamol-induced hepatitis resulted in a 10.7 % decrease in bilirubin level compared to the same indicator in the blood of rats of the III experimental group receiving the combination "silibinin + ademetionine". At the same time, the level of bilirubin in the blood of animals of all experimental groups had statistically significant

TABLE 1

BLOOD BIOCHEMICAL PARAMETERS OF RATS IN A MODEL OF ACUTE PARACETAMOL HEPATITIS

Biochemical indicators	Group I: intact ($n = 10$)	Group II: paracetamol hepatitis ($n = 10$)	Group III: silibinin + ademethionine ($n = 10$)	Group IV: *Hippophae rhamnoides foliae* extract ($n = 10$)	Group V: ademethionine ($n = 10$)	Group VI: *Hippophae rhamnoides foliae* extract + ademethionine ($n = 10$)
AST, U/L	44.30 ± 1.62	66.20 ± 5.94*	59.20 ± 4.24	61.20 ± 2.76	64.00 ± 5.45	57.80 ± 2.12
ALT, U/L	172.16 ± 4.35	209.90 ± 1.07*	186.30 ± 17.37**	197.10 ± 14.78	194.40 ± 3.75	183.80 ± 13.08**
ALP, U/L	657.80 ± 35.07	906.10 ± 26.87*	788.50 ± 7.28**	808.20 ± 42.00**	796.30 ± 29.06**	786.30 ± 20.79**
GGTP, U/L	7.14 ± 0.31	29.5 ± 1.7*	23.7 ± 1.3**	25.1 ± 1.7**	24.2 ± 1.4**	22.3 ± 1.5**

Note. Differences are statistically significant compared to: * – intact group ($p < 0.05$); ** – control group ($p < 0.05$); n – number of animals in each group.

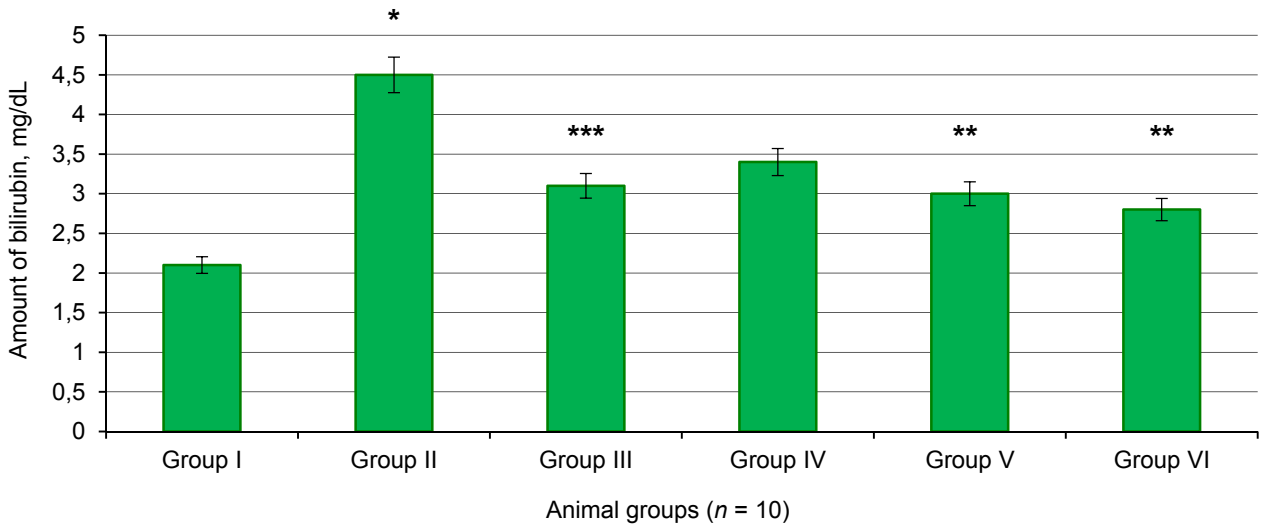

FIG. 1.

*Bilirubin concentration in rat serum in a model of acute paracetamol hepatitis. Differences are statistically significant compared to the values in: * – intact group ($p < 0.001$); ** – control group ($p < 0.05$); n – the number of animals in each group*

differences with the indicators in the control. However, it should be emphasised that the bilirubin level in Group VI rats was restored to the values in intact rats.

DISCUSSION

One of the urgent problems of pharmacology is the search for effective and safe hepatoprotectors for the treatment of drug-induced liver damage. In particular, using a model of acute liver injury paracetamol is similar in pathogenesis to drug-induced hepatitis [15]. According to the literature, hepatotoxic doses of paracetamol lead to centrilobular necrosis and liver failure [16–18].

It follows from the review article devoted to the combined use of drugs by S.V. Okovitiy (2020) that there is currently little information about experimental and clinical studies devoted to the study of the feasibility and possibility of combined use of hepatoprotectors [5, 19].

In a study by I. Giangrandi et al. (2016) it was shown that taking a combination of silymarin (140 mg/day) and ademetionine (200 mg/day) for 12 months (patients with non-alcoholic fatty liver disease) without additional dietary correction led to a decrease in the level of biochemical indicators against the background of regression of liver steatosis [20]. According to A.Y. Au et. al. (2013), "silibinin + ademethionine" combination inhibits both inflammation and oxidative stress by affecting different signalling pathways mediated by nuclear factor NF-B and transcription factor Nrf2 [21].

According to the results of the experiment, it was revealed that the administration of paracetamol produced changes in AST, ALT, ALP, alkaline phosphate, GGTP and total bilirubin activities. Paracetamol and its breakdown products in control animals with toxic hepatitis lead to damage of lipid bilayer of hepatocyte membranes, activation of cytolysis and cholestasis syndromes, disruption of protein, carbohydrate, bioenergetics metabolism and inhibition of enzyme systems of xenobiotics detoxification. When administering the combination of "silibinin + ademethionine", a statistically significant decrease in the level of all studied parameters compared to the control was observed. After administering *Hippophae rhamnoides foliae* extract in the therapeutic regime, hepatoprotective activity was revealed. Thus, the course administration of phytoextract in monotherapy mode causes a decrease in total bilirubin and alkaline phosphatase. It was also revealed that when the liver of rats was damaged by paracetamol and "ademetionine" therapy, the indices of the studied parameters were close to those of the intact group. Correction of the disorders revealed by paracetamol exposure with the combination of "*Hippophae rhamnoides foliae* extract + ademethionine" led to the improvement of rat liver functional status indices, as assessed by the studied markers of liver damage: AST, ALT, GGTP, total bilirubin and alkaline phosphatase. But in comparing all experimental groups of animals treated with different regimens, it is worth noting that the groups of rats that received combinations of drugs had better results compared to rats treated with mono-medications alone. High doses of paracetamol are commonly known to cause necrosis of some liver hepatocytes, and it takes quite a long time for hepatocytes to recover. Nevertheless, administration of the combination "*Hippophae rhamnoides foliae* extract + ademethionine" during the experiment contributed to significant improvement of liver condition in acute paracetamol poisoning.

CONCLUSION

Thus, in toxic hepatitis caused by paracetamol administration, the combined use of "*Hippophae rhamnoides foliae* extract + ademetionine" as well as "silibinin com-

bined with ademetionine", in contrast to "*Hippophae rhamnoides foliae* extract" and "ademethionine" as mono-medications, leads to better recovery of activity level of some biochemical indices of blood serum in experimental animals and thus, as a result, has a more pronounced hepatoprotective effect. The obtained data provide a prerequisite for further research and application of the complex "*Hippophae rhamnoides foliae* extract + ademethionine" for correction of liver diseases caused by various toxic agents.

Conflict of interest

The authors of this article declare no conflicts of interest.

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Information about the authors

Snezhana Yu. Batueva – Postgraduate at the Department of Pharmacology, Clinical Pharmacology with a course of Biochemistry, D. Banzarov Buryat State University, e-mail: bsu.2017@mail.ru, https://orcid.org/0000-0002-0262-3303

Galina P. Lamazhapova – Dr. Sc. (Biol.), Associate Professor at the Department of Pharmacology, Clinical Pharmacology with the course of Biochemistry, D. Banzarov Buryat State University; Head of the Department of Biotechnology, East Siberian State University of Technology and Management, e-mail: lamazhap@mail.ru, https://orcid.org/0000-0002-4118-215X

Yulia A. Kapustina – Cand. Sc. (Biol.), Senior Lecturer at the Department of Pharmacology, Clinical Pharmacology with the course of Biochemistry, D. Banzarov Buryat State University, e-mail: uas.2003@mail.ru, https://orcid.org/0000-0001-9982-5034

Olga A. Rodnaeva – Cand. Sc. (Biol.), Associate Professor at the Department of Pharmacology, Clinical Pharmacology with the course of Biochemistry, D. Banzarov Buryat State University, e-mail: rodnaeva@mail.ru, https://orcid.org/0000-0003-1954-9952

Sergey A. Chukaev – Cand. Sc. (Med.), Associate Professor at the Department of Pharmacology, Clinical Pharmacology with the course of Biochemistry, D. Banzarov Buryat State University, e-mail: s.chukaev@mail.ru, https://orcid.org/0000-0001-7241-9554

EXPERIMENTAL RESEARCHES

CHANGES IN THE PATTERN OF SLEEP DISTURBANCES IN HEALTHY SUBJECTS UNDER 21-DAY ANTI-ORTHOSTATIC HYPOKINESIA

Kovrov G.V. [1],
Vlasova A.V. [1, 2],
Popova O.V. [1],
Chernikova A.G. [1]

[1] State Scientific Center of the Russian Federation – Institute Biomedical Problems, Russian Academy of Sciences (Khoroshevskoe Highway 76A, Moscow 123007, Russian Federation)
[2] I.M. Sechenov First Moscow State Medical University (Sechenov University) (Trubetskaya str. 8 build. 2, Moscow 119991, Russian Federation)

Corresponding author:
Alisa V. Vlasova,
e-mail: vav.vav.va@yandex.ru

ABSTRACT

Background. *Antiorthostatic hypokinesia (ANOH) reproduces some of the effects of weightlessness on the human body and is used to study adaptation to space flight conditions. It is known that ANOH affects nighttime sleep, but there is no information in the literature on the sequence of occurrence of sleep disorders in ANOH.*

The aim. *To study the dynamics of subjective changes in assessing sleep quality under conditions of antiorthostatic hypokinesia.*

Materials and methods. *Six healthy male volunteers (age from 26 to 34 years) participated in the experiment with 21-day ANOH. They were on a medical bed with a body inclination angle relative to the horizon of –6° for 21 days. To assess sleep quality, a structured questionnaire was used that assessed sleep duration, rate of falling asleep, night awakenings, the presence of daytime sleepiness, and daytime falling asleep.*

Results. *Based on the assessment of the dynamics of the sleep efficiency index (SEI), three stages of adaptation were identified. At the stage of acute adaptation (the first 3 days), there is a decrease in SEI from 96.4 to 91.3 ($p < 0.01$), a statistically significant prolongation of falling asleep from 17.6 to 33.6 minutes ($p < 0.01$), an increase duration of night awakenings up to 17.4 minutes, increase in daytime sleepiness by 11 %. In the next 3 days (the "recovery" stage), there is a statistically significant increase in SEI compared to the first stage to 94.7 ($p < 0.01$), but it remains statistically significantly lower than the background values ($p < 0.004$). The number of complaints about daytime sleepiness increases (up to 42 %), evening lights off term shifts later by 26 minutes. At the third stage (the remaining nights) there is a relative stabilization of the sleep-wake cycle.*

Conclusion. *Under conditions of 21-day ANOH, a gradual change in the pattern of sleep disturbances occurs. The most negative changes in terms of subjective assessment were noted in the first three days. Then there is an improvement in falling asleep, a decrease in night awakenings, combined with an increase in daytime sleepiness and the formation of a schedule with later lights off.*

Key words: *antiorthostatic hypokinesia, sleep, space flight, sleep efficiency index*

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ИЗМЕНЕНИЕ ПАТТЕРНА НАРУШЕНИЙ СНА У ЗДОРОВЫХ ЛЮДЕЙ В УСЛОВИЯХ 21-СУТОЧНОЙ АНТИОРТОСТАТИЧЕСКОЙ ГИПОКИНЕЗИИ

Ковров Г.В. [1],
Власова А.В. [1, 2],
Попова О.В. [1],
Черникова А.Г. [1]

[1] ФГБУН Государственный научный центр Российской Федерации – Институт медико-биологических проблем Российской академии наук (123007, г. Москва, Хорошевское шоссе, 76а, Россия)

[2] ФГАОУ ВО «Первый Московский государственный медицинский университет им. И.М. Сеченова» Минздрава России (Сеченовский университет) (119991, г. Москва, ул. Трубецкая, 8, стр. 2, Россия)

Автор, ответственный за переписку:
Власова Алиса Вадимовна,
e-mail: vav.vav.va@yandex.ru

РЕЗЮМЕ

Обоснование. *Антиортостатическая гипокинезия (АНОГ) воспроизводит некоторые эффекты воздействия невесомости на организм человека и применяется для изучения адаптации к условиям космического полёта. Известно, что АНОГ влияет на ночной сон, но в литературе отсутствуют сведения о последовательности возникновения нарушений сна при АНОГ.*

Цель работы. *Изучение динамики субъективных изменений оценки качества сна в условиях антиортостатической гипокинезии.*

Материалы и методы. *В эксперименте с 21-суточной АНОГ участвовали 6 здоровых мужчин-добровольцев (возраст от 26 до 34 лет). Они находились на медицинской кровати с углом наклона тела относительно горизонта –6° в течение 21 суток. Для оценки качества сна был использован структурированный опросник, оценивающий продолжительность сна, скорость засыпания, ночные пробуждения, наличие дневной сонливости, дневных засыпаний.*

Результаты. *На основании оценки динамики индекса эффективности сна (ИЭС) было выделено 3 этапа адаптации. На этапе острой адаптации (первые 3 суток) происходит снижение ИЭС с 96,4 до 91,3 ($p < 0,01$), статистически значимое удлинение засыпания с 17,6 до 33,6 мин ($p < 0,01$), увеличение продолжительности ночных пробуждений до 17,4 мин, усиление дневной сонливости на 11 %. В следующие 3 суток («восстановительный» этап) отмечается статистически значимое увеличение ИЭС по сравнению с 1-м этапом до 94,7 ($p < 0,01$), но он остаётся статистически значимо ниже фоновых значений ($p < 0,004$). Возрастает количество жалоб на дневную сонливость (до 42 %), сроки вечернего отбоя смещаются позже на 26 минут. На 3-м этапе (оставшиеся ночи) происходит относительная стабилизация цикла «сон – бодрствование».*

Заключение. *В условиях 21-суточной АНОГ происходит постепенное изменение паттерна нарушений сна. Наиболее негативные в плане субъективной оценки изменения отмечались в первые 3 дня. Затем отмечается улучшение засыпания, снижение ночных пробуждений в сочетании с увеличением дневной сонливости и формированием режима с более поздним отбоем.*

Ключевые слова: *антиортостатическая гипокинезия, сон, космический полёт, индекс эффективности сна*

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INTRODUCTION

Sleep has a role in supporting psychophysiological homeostasis at the proper level and plays an important role in the level of physical and mental performance [1]. Astronauts have reported sleep disturbances during spaceflight, which become less with the time spent in space [2].

The effect of spaceflight conditions that affect human sleep is also studied in ground-based modelling experiments. To be specific, head-down bed rest (HDBR) reproduces some of the effects caused by weightlessness on the human body and has been used to study adaptation to spaceflight conditions [3]. HDBR is an exposure that disrupts nighttime sleep. Staying for a long time with the head tilted downward reduces intracranial perfusion and leads to stasis in the jugular vein [4, 5], decreased systolic blood pressure and decreased heart rate [6], and increased intracranial pressure [7]. Body position also has an effect on liquor outflow and amyloid elimination activity [8]. It was previously shown with the use of polysomnography that under HDBR conditions there is a lengthening of the latent periods of stages 1, 2 and 3 of sleep, an increase in the duration of nocturnal awakenings and stage 2, as well as a decrease in the duration of stage 3 of sleep, the stage with rapid eye movements [9]. Studying sleep during the first day of hypokinesia has shown that there is a decrease in the stage 3 of sleep, the stage of sleep with rapid eye movements, an increase in stage 1, as well as a deterioration in the subjective quality of sleep [10, 11]. The other study observed a decrease in stage 4 sleep and an increase in the frequency of awakenings during experimental bed rest when the head was tilted downwards by –6° [12, 13].

In a single study, the authors have observed that during the first 3 days of HDBR, nocturnal sleep, level of daytime vigour and psychophysiological functions were not impaired, although a slight deterioration of attention function was observed in the morning [13].

In HDBR conditions, along with deterioration of sleep quality, daytime symptoms of circadian rhythm disturbance – the appearance of sleepiness, development of physical and mental fatigue are also observed [14].

An absence of any evidence in the literature about the consistency in the occurrence of sleep disturbances during HDBR determined the purpose of our work as a study of the dynamics of subjective changes in the assessment of sleep quality under conditions of head-down bed rest.

MATERIALS AND METHODS

The study is an open pre-experimental, prospective research. It was undertaken 5 days before HDBR (baseline assessment) and daily under HDBR conditions in the evening (for 21 days). Six practically healthy male volunteers aged 24 to 40 years (mean age – 29.8 ± 4.6 years) with body weight 75.2 ± 8.8 kg, body length 177.8 ± 5.3 cm, body mass index (BMI) 23.8 ± 2.7 kg/m^2 were enrolled in the study. All participants had undergone medical selection by the medical expert commission of the State Science Center of the Russian Federation – Institute of Bio-Medical Problems of the Russian Academy of Sciences, during which no diseases and pathologies preventing participation in the experiment were found. Each participant had signed a voluntary informed consent to participate in the experiment before being enrolled in the study. The study programme has been approved by the Biomedical Ethics Commission of the State Scientific Centre of the Russian Federation – Institute of Bio-Medical Problems of the Russian Academy of Sciences (SSC RF – IBMP RAS) (Protocol No. 621 dated August 8, 2022). The inclusion criteria for the study were the conclusion of the medical expert committee and consent to participate in the study.

The study was conducted on the basis of the SSC RF – IBMP RAS. Volunteers were kept in an anti-orthostatic position on a medical bed with a body angle relative to the horizon of –6°, without exercise and with moderate restriction of movement for 21 days.

Environmental factors: according to the study cyclogram, lights were turned off after 23:00. Additionally, the gadgets remained close to the participants at all times, but their use during the night was not monitored.

The study assessed data from questionnaires conducted 5 days before HDBR and daily under HDBR conditions in the evening (for 21 days). A structured questionnaire (non-validated) was specifically designed to identify sleep and wakefulness patterns, where participants answered questions related to sleep quality and sleep-wake cycle patterns in the evening. At its core were questions reflecting basic characteristics of sleep quality (sleep duration, rate of falling asleep, nocturnal awakenings) and wakefulness activity (presence of daytime sleepiness, daytime falling asleep, circadian distribution of activity). The questionnaire was not validated. The questionnaire is presented in Figure 1 (non-validated).

From the questionnaire data obtained, a sleep efficiency index (SEI) [1] was calculated as the ratio of the time from lights off term to final awakening to the same time minus the duration of falling asleep and nocturnal awakenings. The rest of the answers to the questions were scored. Responses reflecting worsening sleep and wakefulness quality corresponded to an increase in point estimates.

Statistical processing was performed using Statistica 10.0 program (StatSoft Inc., USA). In this analysis, the rank, quantitative (non-normal distribution) nonparametric Mann – Whitney test was used to compare unrelated groups.

RESULTS

A survey of trial participants revealed that 5 out of 6 healthy volunteers were bothered by long periods of falling asleep and night wakings during the first days of the experiment. Sleep disorders were associated by the participants with uncomfortable sleeping conditions and changes in the horizontal axis of the body, redistributing body flu-

ids. In the following days at HDBR, their sleep problems became less of a problem.

Based on questionnaire results during the entire HDBR, all volunteers episodically reported difficulty falling asleep, nocturnal awakenings, increased daytime sleepiness accompanied by daytime sleepiness and drowsiness.

A study of sleep patterns based on a questionnaire we had designed revealed that during the 21-day HDBR period, the trial participants established a regime with a statistically significant later lights off and later morning awakening compared to background studies. The data are summarised in Table 1.

When the questionnaire has been analysed, it was found that under HDBR conditions, falling asleep for more than 30 minutes was observed to occur during 10 % of nights; in 24 % of cases, the time to fall asleep was between 15 and 30 minutes, and only 66 % of cases, the duration of falling asleep remained within the normal range (quick guide).

In analysing the number of awakenings within the HDBR condition, it was found that 10 % of the participants reported nocturnal awakenings lasting more than 30 minutes, and 90 % of the participants slept without prolonged awakenings.

Sleep
Sleep-related questions refer to past sleep.
1. What time did you go to bed?
2. How long did it take you to fall asleep?
   Up to 15 minutes
   Up to 30 minutes
   Up to 1 hour
   More than an hour
3. Waking up at night for more than 30 minutes
   0 times
   1 time
   2 times
   More than 2 times
4. What time did you finally wake up?

Wakefulness
Wakefulness questions refer to the past day.
1. Have you had any drowsy states or daytime naps?
   0 times
   1 time
   2 or more times
2. Have you had any daytime sleepiness?
   Yes
   No
3. Your activity during the day was higher
   Until 12:00.
   12 to 5:00 p.m.
   From 5:00 p.m.

FIG. 1.
Structured "Sleep – wake" questionnaire (non-validated)

TABLE 1
MAIN ANALYZED INDICATORS OF THE "SLEEP – WAKE" QUESTIONNAIRE

Indicators	Background data	HDBR data	*p*
Lights off (astronomical time)	23:35	00:47	0.001
Morning awakening (astronomical time)	07:25	07:52	0.01
Sleeping time (excluding falling asleep and waking time during night awakenings), h	7:08	7:15	0.90
Sleep efficiency index, %	96.43	94.39	0.26
Falling asleep time, h	0:29	0:40	0.37
Representation of drowsiness, %	33	63	0.044

An assessment of daytime wakefulness patterns revealed that throughout the HDBR, participants reported increased daytime sleepiness with occasional drowsiness. The presence of sleepiness and daytime falling asleep during wakefulness was noted in 61 % of cases, and in 8 % of cases daytime falling asleep could be more than 2 times per day.

SEI values, time to fall asleep, wakefulness time during nocturnal awakenings, and daytime sleepiness were used to assess sleep quality. The dynamics of these indicators is summarised in Figure 2.

The worst night was the first night of the HDBR stay. Compared to background data 5 days before HDBR, sleep on the first day was characterised by a decrease in SEI from 95 to 84 % ($p = 0.013$), a statistically significant prolongation of falling asleep from 17.55 to 42.1 min ($p = 0.050$), and an increase in the duration of nocturnal awakenings from 4.8 to 34.8 min.

Three stages of adaptive changes in the sleep-wake cycle in a 21-day HDBR condition were revealed based on visual assessment of SEI dynamics, time to fall asleep, duration of nocturnal awakenings, and daytime sleepiness.

Table 2 summarises the mean values of some indicators reflecting the quality of sleep and wakefulness for the highlighted stages.

Stage 1 – stage of acute adaptation, where during the first 3 days there is a decrease in SEI indices from 96.4 to 91.3 % ($p < 0.01$), statistically significant prolongation of falling asleep from 17.6 to 33.6 min ($p < 0.01$), increase in the duration of nocturnal awakenings up to 17.4 min; there is no increase in daytime sleepiness.

Stage 2 – recovery stage (next 3 days), where SEI statistically significantly increased compared to stage 1 up to 94.7 % ($p < 0.01$) but remained statistically significantly less than it was in the background ($p < 0.004$). The number of cases of daytime sleepiness indices increases by 38 %, the evening lights off time becomes even later by 26 min.

Stage 3 – the stage of relative stabilisation of the sleep-wake cycle (remaining nights). It is observed here the latest time of lights off – at 1 a.m. ($p < 0.003$) – in comparison with all stages of adaptation and background indicators (lights off time in the background – 23:36, in stage 1 – 23:54, in stage 2 – 00:20); the latest awakening time was at 8 a.m. (08:03) (in the background – 07:16, in stage 1 – 07:34, in stage 2 – 07:31), while sleep quality indicators (falling asleep time, night awakenings, SEI) and daytime sleepiness did not differ compared to background.

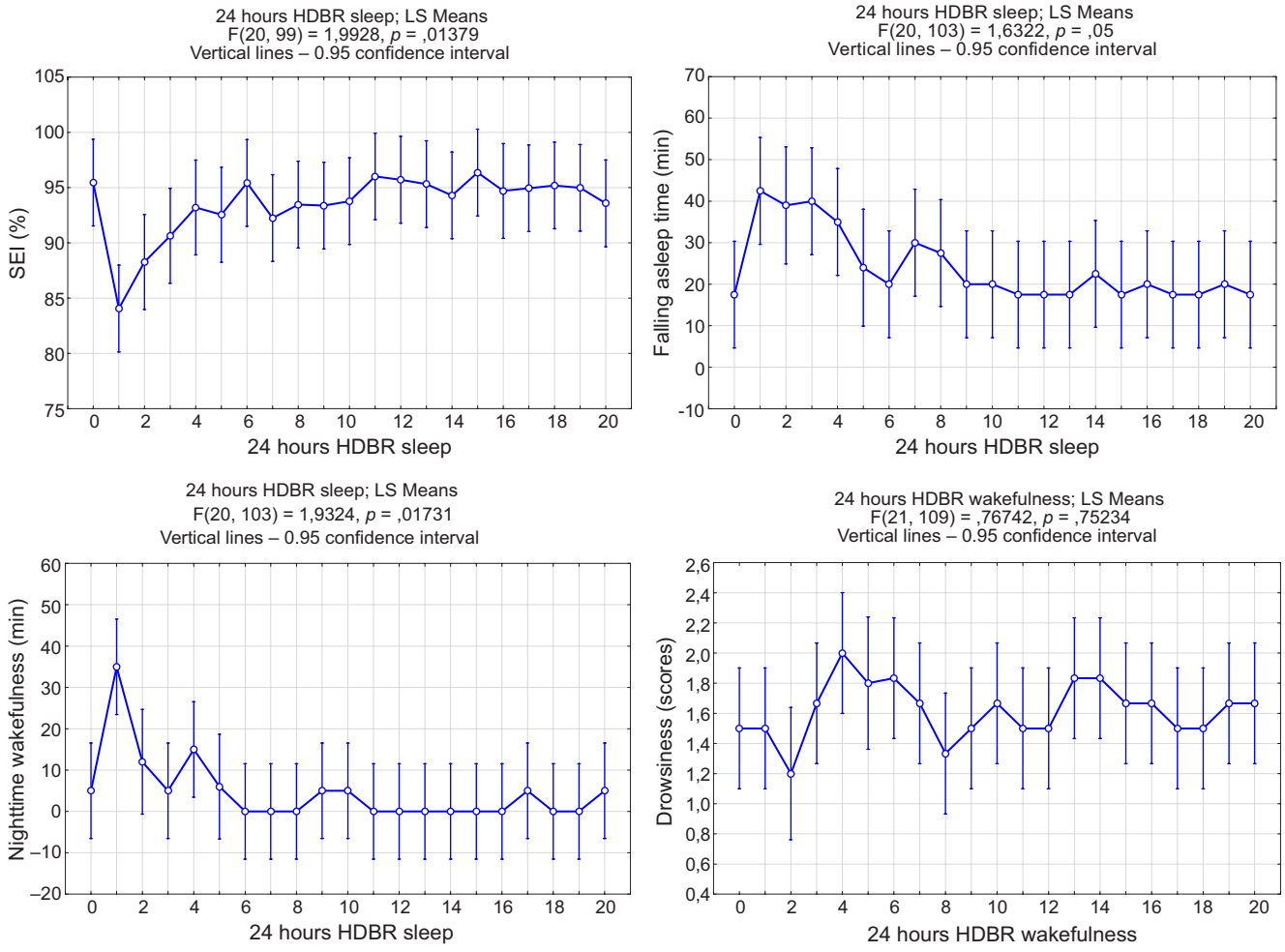

FIG. 2.

Changes in sleep and wakefulness characteristics under 21-day HDBR conditions

TABLE 2

STATE OF "SLEEP – WAKE" CYCLE INDICATORS AT DIFFERENT STAGES OF ADAPTATION ACCORDING TO QUESTIONNAIRE RESULTS

HDBR stages	Background	1st stage	2nd stage	3st stage
Lights off (astronomical time)	23:36	23:54	00:20	01:00
Falling asleep time, min	17.55	33.6	24.6	19.8
Wakefulness time during awakenings, min	4.8	17.4	6.9	0.09
Lights On (astronomical time)	07:16	07:34	07:31	08:03
Time in bed, h	7.63	7.66	7.15	7.04
Presentation of drowsiness, % of cases	33	44	82	62
SEI, %	96.33	91.32	94.73	94.91

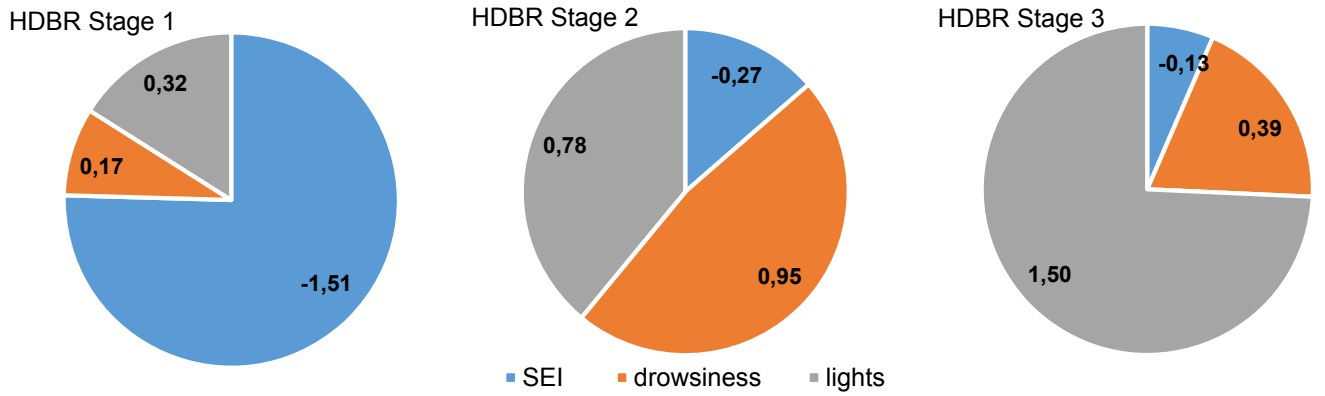

FIG. 3.
Differences of sleep-wake cycle indicators with background in standardised units at different stages of HDBR

Since the indicators are multi-dimensional, a data standardisation procedure was undertaken where the sample mean of the indicator is assumed to be 0 and its standard deviation is assumed to be 1. Differences with background values for all selected HDBR stages were calculated from standardised data. The results are summarised in Figure 3. The presented figure shows that in the acute period of adaptation the greatest changes are associated with a decrease in the sleep efficiency index, in stage 2 – with a shift in lights off time and an increase in daytime sleepiness, in stage 3 – with a shift in lights off time to later.

DISCUSSION

The interest in studying the particularities and timing of adaptation of somnogenic mechanisms to existence in extreme conditions is of definite importance, being able to predict the features of sleep in different periods of adaptation [2]. The results obtained allowed this study to identify successive stages of circadian system adaptation to HDBR conditions. The selected stages and their timing are conditional, as this selection was made on the basis of visual assessment of some indicators of the questionnaire describing the peculiarities of the sleep-wake cycle. Statistically significant differences have been revealed between these stages, but it is not entirely certain that the adaptation time stages that were defined are definitive, and further studies may present the time required for habituation to HDBR conditions in a different way. There is sufficient variation in SEI indices even during the period of acute adaptation (during the first 3 nights). Whereas on the first, worst night, SEI values are only 84 % (normal SEI should be more than 85 %), the following two nights the quality of sleep begins to recover – the average SEI value is 92 %. For this reason, it is possible that the acute adaptation phase may comprise only a single night and the remaining days may be classified as the sub-acute recovery phase.

Assessment of the state of the sleep-wake cycle at stage 2 of adaptation (recovery stage) and analy-

sis of its clinical manifestations is an important component of the performed study. The gradual improvement in sleep quality during the recovery phase was combined with an unexpected finding of a 38 % increase in episodes of daytime sleepiness. The diagnosis of insomnia in clinical medicine is based not only on the assessment of sleep quality, but also on the assessment of wakefulness quality, including daytime sleepiness, as a characteristic indicating the negative impact of poor sleep quality on subsequent wakefulness [15]. This raises the suggestion that the increase in daytime sleepiness with improved sleep quality and unchanged sleep duration is a characteristic independent of sleep quality but possibly reflects an increased need for nocturnal sleep on days 4–6.

The remaining nights associated with stage 3 of relative adaptation were closest to background values. The assessment of this stage showed that changes in the sleep-wake cycle consisted in approaching the background values of the parameters that were assessed, which is a positive sign of adaptation. The fact that there is a significant shift in lights off and morning rise and fall to later hours during the relative stabilisation phase appears to be of particular interest. A shift to later bedtime is often revealed in healthy individuals under different stressful situations [16, 17]. It is assumed that the most likely reason for the development of later lights off time is probably caused by features of prior wakefulness (low physical activity). Ultimately, shifting the lights off time results in a later rise. The phenomenon of regime shift to later dates appears to be an important factor posing a potential threat to circadian rhythm stability in general [18].

Apart from the findings that were highlighted about the stages of adaptation, a number of shortcomings of the study have also been revealed, which impose a number of limitations. These include the small size of the group and the use of subjective assessments of the quality of the sleep-wake cycle.

CONCLUSION

Under HDBR conditions, there is a sequential occurrence of various circadian rhythm disorders: a decrease in sleep efficiency index as a result of lengthening of falling asleep and the appearance of nocturnal awakenings, followed by the development of hypersomniac symptoms (daytime sleepiness) against the background of improved nocturnal sleep, and then a gradual shift of the sleep regime to later periods. At different stages of circadian rhythm adaptation to HDBR conditions, the holistic picture of insomniac disorders changes, constituting a pattern of sleep disorders specific for each stage in healthy people under conditions of 21-day head-down bed rest.

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Conflict of interest

The authors of this article declare no conflicts of interest.

Ethical review

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Information about the authors

Gennady V. Kovrov – Dr. Sc. (Med.), Professor, Senior Research Officer, State Scientific Center of the Russian Federation – Institute Biomedical Problems, Russian Academy of Sciences, e-mail: kgv2006@yandex.ru, https://orcid.org/0000-0002-3564-6798

Alisa V. Vlasova – Student, N.V. Sklifosovsky Institute of Clinical Medicine, I.M. Sechenov First Moscow State Medical University (Sechenov University); Research Assistant, State Scientific Center of the Russian Federation – Institute Biomedical Problems, Russian Academy of Sciences, e-mail: vav.vav.va@yandex.ru, https://orcid.org/0009-0007-7901-8869

Olga V. Popova – Junior Research Officer, State Scientific Center of the Russian Federation – Institute Biomedical Problems, Russian Academy of Sciences, e-mail: olya.popovaolga2710@yandex.ru, https://orcid.org/0009-0002-3749-588X

Anna G. Chernikova – Cand. Sc. (Biol.), Senior Research Officer, State Scientific Center of the Russian Federation – Institute Biomedical Problems, Russian Academy of Sciences, e-mail: anna.impb@mail.ru, https://orcid.org/0000-0002-2596-8929

ERRATUM

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Address: Bortsov Revolutsii str. 1, Irkutsk 664003, Russian Federation.
Tel.: (3952) 29-03-37, 29-03-70. E-mail: arleon58@gmail.com