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THE SPECIFICS OF ENCEPHALITIS AFTER COVID-19

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Encephalitis is a group of acute infectious diseases affecting the substance of the brain. They often lead to disability or death, and, therefore, require urgent medical attention. The article discusses the etiology, pathogenesis, and clinical picture of encephalitis, with special attention to the course of this disease after the COVID-19 pandemic. We note the growing number of encephalitis cases, especially of autoimmune variety and those caused by herpes. The possible reason behind this trend is the disruption of operation of the immune system brought by COVID-19, which manifests as a cytokine storm, neuroinflammation, and autoimmune reactions. There are cases of COVID-19-dependent encephalitis described. The pathways taken by SARS-CoV-2 to penetrate into the cells of the central nervous system have not yet been fully studied, although there are hypotheses that this happens both trans-synaptically through mechanoreceptors and chemoreceptors of the respiratory system into the medulla oblongata, and through receptors of the angiotensin converting enzyme 2.

Keywords: encephalitis, COVID-19, neuroinfection, autoimmune encephalitis

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ОСОБЕННОСТИ ТЕЧЕНИЯ ЭНЦЕФАЛИТОВ ПОСЛЕ ПЕРЕНЕСЕННОЙ НОВОЙ КОРОНАВИРУСНОЙ ИНФЕКЦИИ COVID-19

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Энцефалиты представляют собой группу острых инфекционных заболеваний, поражающих вещество головного мозга. Они часто приводят к инвалидности или летальному исходу и в связи с этим требуют неотложной медицинской помощи. В статье рассмотрены этиология, патогенез и клиническая картина энцефалитов. Особое внимание уделено течению энцефалитов после пандемии COVID-19. Отмечен рост числа энцефалитов, особенно среди аутоиммунных энцефалитов, энцефалитов, вызванных герпес-вирусами. Вероятно, эта тенденция связана с тем, что взаимодействие вируса COVID-19 с организмом приводит к нарушению работы иммунной системы, что проявляется развитием цитокинового шторма, нейровоспалением и развитием аутоиммунной реакции. Описаны случаи развития COVID-19-зависимого энцефалита. Механизмы проникновения вируса COVID-19 в клетки центральной нервной системы еще не до конца изучены, хотя и существуют гипотезы, что это происходит как транссинаптическим путем через механорецепторы и хеморецепторы респираторной системы в продолговатый мозг, так и через рецепторы ангиотензинпревращающего фермента 2.

Ключевые слова: энцефалит, COVID-19, нейроинфекция, аутоиммунный энцефалит

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Encephalitis is an acute inflammation of the brain tissue [1]. The urgency of discussion of this subject is substantiated by the severe course of the disease, need for emergency medical care, sometimes without delays, and the possible disability or lethal outcomes [2]. Understanding the etiology, links of pathogenesis, and knowing the clinical picture of encephalitis, a clinician can correctly diagnose the condition and initiate the necessary therapy.

Among neuroinfections, the share of encephalitis varies from 3.8 to 65%; this wide a range can probably be explained by the epidemiological situation in a given region and availability of advanced laboratory and diagnostic equipment therein [3–5]. Encephalitis is a polyethological disease, but its most common pathogens are viruses [5, 6]: they make up to 90% of all cases [4]. The prevailing varieties thereof are the tick-borne encephalitis (TBE) virus, enteroviruses (various strains of Coxsackieviruses and ECHO viruses), and herpes virus [7].

Autoimmune encephalitides (AIE) form a group of their own. They are characterized by an autoimmune inflammatory process in the brain and production of antibodies to extracellular or intracellular structures of the central nervous system [8]. The most common AIE is anti NMDAR encephalitis [9]. The known

reasons triggering autoimmune process in the context of this diseases are neoplasms and herpetic encephalitis [10].

The clinical picture of encephalitis includes a general infectious syndrome (weakness, fever, myalgia, arthralgia), a cerebral syndrome (nausea, vomiting, dizziness), and focal symptoms [11]. Depending on the cause, the prevailing conditions may be flaccid paralysis of upper limbs and neck (associated with tick-borne encephalitis) [12], oculomotor disorders (Von Economo encephalitis) [13], or mental disorders (autoimmune encephalitis) [14].

The purpose of this literature review is to analyze the course of encephalitis against the background of the new coronavirus infection, and to compare the respective data with those describing the pre-pandemic period.

Main part

The state of the person's immune system plays an important role in the pathogenesis of various forms of encephalitis. Over the past 3 years, the world has seen the COVID-19 pandemic brought by SARS-CoV-2.

In addition to the damage to respiratory system, COVID-19 caused extrapulmonary complications under the influence of several factors: a long-lasting inflammation; persistence of the virus or parts thereof in organs with possible reactivation of inflammation; production of antibodies that cross-respond with body tissues; development of coagulopathies [15]. The growth of neurological complications, including encephalitis, is natural. One of the pathways of damage to the central nervous system (CNS) may be through the link between SARS-CoV-2 and receptors of angiotensin-converting enzyme 2 (ACE 2), which are common in neurons and glial cells of the CNS [16]. Another considered pathway involves transsynaptic penetration into medulla oblongata through mechanoreceptors and chemoreceptors found in the lungs [17].

The analysis of data from 23 sources, which covered about 130,000 COVID-19 patients, showed that the proportion of patients with encephalitis is about 0.215%, while mortality is 13.4% [18]. Among all patients with neurological symptoms, the share of encephalitis ranges from 13 to 40% [19]. Neuroimaging scans of 127 patients revealed the following: 86 patients had nonspecific COVID-19-associated encephalitis; 13 — acute demyelinating encephalomyelitis; 4 — acute necrotic encephalopathy; 9 — limbic encephalitis; 5 — Bickerstaff brainstem encephalitis; 13 — encephalitis with focal or diffuse leptomeningeal disorders; 26 — concomitant encephalopathy and encephalitis with other clinical and morphological findings [19].

The symptoms registered in patients with encephalitis during the acute phase of COVID-19 were seizures (29.5%), confusion (23.2%), headache (20.5%), disorientation (15.2%), and a change in mental status [20]. In over half of the cases considered, laboratory examination revealed changes visible on MRI scans, EEG records, and in composition of the cerebrospinal fluid [20, 21].

COVID-19-associated encephalitis can develop a few weeks after the acute phase of the disease. A clinical case report [22] describes acute hemorrhagic leukoencephalitis in a 46-year-old patient who, after hospitalization with confirmed COVID-19, was discharged for quarantined treatment at home. Five weeks later, he was urgently taken to the hospital with complaints of headache and impaired consciousness. His neurological status included depression of consciousness (up to 11 points on the Glasgow Coma Scale), upper left limb plegia and lower left limb paresis (up to 3 points), while the tendon reflexes were preserved. Computed tomography revealed multifocal non-hemorrhagic lesions in both hemispheres of the brain, MRI — lesion of white matter in the bilateral frontal, parietal lobes, left thalamus, left cerebral peduncle and medulla oblongata. Lymphocytic pleocytosis with increased protein content was observed in the cerebrospinal fluid. The patient was prescribed intravenous administration of 1 g of methylprednisolone per day for 5 days. After 5 days, against the background of deterioration of the patient's condition, new MRI scans were made, and they showed greater number of lesions, now hemorrhagic, and edema with trunk wedging. The treatment plan was extended with reinforced decongestive therapy and a trepanation, but the patient died on the same day. A meta-analysis of the reported cases of acute hemorrhagic leukoencephalitis showed that their amount has grown compared to the pre-pandemic period, and the number of the associated deaths was up to 32% [23].

Herpes encephalitis (HE) is one of the most common varieties of the disease [24]. Typically, infection with a herpes virus occurs at an early age. It penetrates into the cell, release proteins, viral DNA, and begins production of new viral units [25]. Immune system triggers cellular immunity, which involves activation of CD8+ T cells, differentiation of CD4 cells into T helpers, production of humoral immunity elements (IFN-γ, IL-2, TNF-α), and activation of B lymphocytes. As a result, virus replication slows down and it becomes latent, persisting in sensory and sympathetic ganglia. A possible pathway to development of HE is retrograde transport of virus particles along the fibers of the olfactory or trigeminal nerves [26]. With COVID-19 in the background, CD 8- and CD 4- cells are depleted, gamma interferon production slows down, which probably leads to increased replication of the herpes virus and subsequent development of HE [25, 26].

Another consequence of the immune system's reaction to SARS-CoV-2 is cytokine storm. Some researchers believe that cytokine storm is a factor in the development of AIE in patients who had COVID-19 [27, 28]. A meta-analysis revealed that the most common type of the disease is limbic encephalitis (37%), followed by anti NMDAR encephalitis (26%). There were cases of encephalitis registered in vaccinated patients, with 38.5% of them having AstraZeneca vaccine, 33.8% — Pfizer vaccine, and 16.9% — Moderna vaccine [29, 30]. The mechanism of development of this condition after vaccination remains unclear. Russian vaccines, designed with the negative experience factored in, were not found to be associated with encephalitis, therefore, in our opinion, they can be the best recommendation for prevention of COVID-19 [31].

Influenza can develop complications in the form of influenza-associated encephalitis [5]. It was reasonable to expect that in the epidemic season of 2022–2023, in a population whose immunity has been weakened and modified by repeated infection with SARS-CoV-2, there will be more cases of damage to the nervous system done by the influenza virus. A group of researchers examined a Romanian cohort of children aged 1–6 years (n = 301), comparing the frequency of such cases with that registered during the previous epidemic seasons. They found that the 2022–2023 flu season was characterized by a large number of coinfections (viral, bacterial, fungal, and parasitic), which were more severe, with longer hospital stays and more complications ($p < 0.05$); moreover, the patients received oxygen therapy for significantly longer periods of time ($p < 0.05$), and none of them was vaccinated against influenza [32]. The researchers concluded that a history of COVID-19 aggravates flu, at least in minors, especially among young children who are more prone to developing serious complications. The second

conclusion of this study is a recommendation to encourage the widest possible flu vaccination.

The data obtained are of great interest from a fundamental point of view: it is well known that during the COVID-19 pandemic, the incidence of influenza significantly decreased throughout the world [33]. Moreover, the curve of incidence of all other airborne infections dropped [34, 35], and there were similar trends registered for infections transmitted otherwise (in particular, HIV and hepatitis B) [36]. SARS-CoV-2 was assumed to actively suppress circulation of other infectious agents during the pandemic, but currently, we are witnessing a return of other nosological forms, and, as we tried to highlight in this work, there are noticeable changes in their patterns and character of damage to the CNS.

There is no doubt that any encephalitis should be treated immediately. The common approach is to identify its etiology, cause, and begin etiotropic treatment [11, 37–40], adding pathogenetic and symptomatic therapy. It is also necessary to account for the possibility of a more severe course of the disease in people with a history of COVID-19.

CONCLUSION

Encephalitis is a catastrophic condition that can lead to death. Timely diagnosis and adequate therapy improves the prognosis for patients. In recent years, there has been an increase in the number of encephalitis cases, including its autoimmune varieties, and the amount of lethal outcomes therefrom has also grown. It is not always possible to identify clinical and diagnostic signs of encephalitis, and the clinical picture may be blurred or interpreted as a manifestation of another nosology. The COVID-19 pandemic and the specifics of its course, including effects on the immune system, cytokine storm, and subsequent development of long COVID, are the likely factors conditioning the growing frequency of encephalitis. The mechanisms of SARS-CoV-2 penetration into cells and the ways the virus interacts with the nervous system remain partially unknown, but it is certain that encephalitis concomitant with COVID-19 worsens the patient's prognosis. Further investigation of this issue and the development of treatment protocols will contribute to prevention of complications and lethal outcomes.

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MODERN APPROACHES TO ASSESSMENT OF MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA (PLASMA CELL MYELOMA) CASES

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The treatment of multiple myeloma is inextricably linked to the need for assessment and monitoring of the minimal residual disease (MRD). Assessment of the MRD allows evaluating the efficacy of therapy and obtaining significant prognostic information; it is an indicator of the degree of eradication of the tumor clone. The methods for detecting residual tumor cells evolve constantly, which translates into updates of the criteria reflecting the scale of response to therapy. There is no single MRD detection technique; common recommendations suggest seeking for pathological cells both intramedullary and extramedullary. This review describes current MDR determination methods, including imaging, next generation multiparametric flow cytometry, and methods based on DNA analysis — allele-specific oligonucleotide polymerase chain reaction and next generation sequencing. We compare their advantages, limitations, disadvantages, clinical significance, and show the necessary sensitivity thresholds of the described methods and the conditions that make this or that approach ideal in the context of detection of MRD.

Keywords: multiple myeloma, minimal residual disease, methods of assessment, flow cytometry, next generation sequencing

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СОВРЕМЕННЫЕ ПОДХОДЫ К ОЦЕНКЕ МИНИМАЛЬНОЙ ОСТАТОЧНОЙ БОЛЕЗНИ ПРИ МНОЖЕСТВЕННОЙ МИЕЛОМЕ (ПЛАЗМОКЛЕТОЧНОЙ МИЕЛОМЕ)

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Лечение множественной миеломы (ММ) неразрывно связано с необходимостью оценки и мониторирования минимальной остаточной болезни (МОБ). Определение МОБ является важной задачей, позволяющей более глубоко оценить эффективность терапии, получить значимую прогностическую информацию, и является определяющим критерием степени эрадикации опухолевого клона. Это обусловливает необходимость совершенствования методов выявления остаточных опухолевых клеток и приводит к обновлению критериев определения глубины ответа в соответствии с уровнем МОБ. В настоящее время не существует единого метода обнаружения МОБ, рекомендуется использовать как интрамедуллярную, так и экстрамедуллярную детекцию патологических клеток. В обзоре описаны современные методы определения МОБ, включая методы визуализации, выявление остаточных опухолевых клеток в образцах костного мозга и периферической крови с использованием многопараметрической проточной цитометрии (МПЦ), в том числе нового поколения (NGF), и методы, основанные на анализе ДНК — аллель-специфичная олигонуклеотидная полимеразная цепная реакция (АСО-ПЦР) и секвенирование нового поколения (NGS). Проведен сравнительный анализ их преимуществ, ограничений, недостатков и, соответственно, клинической значимости. Показаны необходимые пороги чувствительности описываемых методов и ситуации, в которых применение того или иного метода является оптимальным для диагностики МОБ.

Ключевые слова: множественная миелома, минимальная остаточная болезнь, методы оценки, проточная цитометрия, секвенирование нового поколения

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Multiple myeloma (MM) is a B-cell malignant tumor, the morphological substrate of which are plasma cells producing monoclonal immunoglobulin. In 2017, World Health Organization (WHO) replaced "multiple myeloma" with "plasma cell myeloma" in its registers. However, in the context of the 5th Edition of the World Health Organization Classification of Hematolymphoid Tumors (2022), experts discussing mature lymphoid and histiocyte-dendritic cell neoplasms strongly supported the term "multiple myeloma" rather than "plasma cell myeloma," and thus it was adopted in the International Consensus Classification of Mature Lymphoid Neoplasms [1]. Therefore, in this article, we call the considered disease "multiple myeloma," as is habitual for hematologists.

It is generally recognized that monitoring of minimal residual disease (MRD) in multiple myeloma cases, which aims at detecting subclinical amounts of myeloma cells after successful antitumor therapy, is an important task that allows a more in-depth assessment of the said therapy's efficacy, adds significant prognostic information regarding overall survival (OS) and progression-free survival (PFS) of MM patients, and yields data needed to establish the degree of eradication of the tumor clone. In this connection, improvement of the methods for detecting residual tumor cells is a continuous effort, and the categories of degree of response in accordance with the MRD level are being constantly updated [2–4].

In recent years, MRD detection methods have been developing rapidly, and their sensitivity and applicability have expanded significantly. To improve the sensitivity of myeloma cell detection, there were developed new high-performance bone marrow (BM) aspirates evaluation methods, including multiparametric flow cytometry (MFC), allele-specific oligonucleotide qualitative polymerase chain reaction and next-generation sequencing (NGS). These methods enable quick examination of several thousands to a million BM cells or the

corresponding amount of DNA in a single test, thus quantifying the residual tumor cells in BM.

It is known that MRD-negative (MRD(–)) patients will inevitably relapse, and in some of them, neither MFC nor PCR can detect tumor cells, which supports the need for further efforts to standardize and improve MRD diagnostics.

A lower MRD detection cutoff value peculiar to the sensitive types of examination, such as NGS or highly sensitive MFC, will further improve disease prediction capabilities [5, 6]. For example, using NGS and allocating patients into 3 groups by time to progression (TTP), a group of researchers has shown that people with high (< 10^{-3}), intermediate (10^{-3}–10^{-5}), and low (> 10^{-5}) levels of MRD can have significantly different TTP (27, 48 and 80 months, respectively) [5]. Thus, currently, 10^{-5} is the threshold for affirmation of an MRD-negative status.

In 2016, the International Myeloma Working Group (IMWG) published the following MRD(–) status criteria [7]:

- persistent MRD(–) status, i.e., MRD negative results of BM cells examinations with NGF and/or NGS and PET-CT, persisting for 1 year;
- MRD(–) status confirmed by flow cytometry, that is, absence of aberrant phenotype clonal plasma cells (PCs) in BM aspirates according to NGF that follows the standard EuroFlow operating procedure (or an equivalent validated protocol), minimum sensitivity of 10^{-5} or higher;
- MRD(–) status confirmed by sequencing, i.e., absence of clonal PCs in the results of NGS of BM aspirates, with clone presence defined as less than two identical readings in BM aspirates' DNA sequences established with a minimum sensitivity of 10^{-5} or higher;
- MRD(–) status confirmed by NGF or NGS plus disappearance of each area of increased absorption of the marker that was detected initially or by previous PET-CT, or a drop of the number thereof below the mediastinum SUV value, or below normal.

This review aims to comparatively analyze the advantages, limitations, disadvantages, and clinical significance of the current MRD assessment methods, and describe conditions making this or that method optimal in a given clinical situation.

MRD assessment methods in multiple (plasma cell) myeloma cases

Serological methods of identification of tumor clone

In MM cases, tumor load is diagnosed and monitored through identification of free light chains (FLC) in serum and urine [8]. Currently, assessment of serum FLC κ and λ is one of the routine tests, especially for patients with nonsecretory and oligosecretory myeloma and AL-amyloidosis [9].

Back in 2006, IMWG group included normalization of the FLC level and absence of clonal myeloma cells in BM biopsies sampled from MM patients, as established by immunohistochemistry and/or immunofluorescence, in the list of more stringent criteria defining complete response (CR) [10]. In diagnostics, FLC ratio is an independent prognostic factor of aggressiveness of the disease [11], which also helps stratify patients into risk groups [12]. However, there is still no single opinion about inclusion of the FLC test into routine monitoring of MRD in MM patients, because some studies report contradictory results, even in the context of treatment response [13, 15]. For example, it was shown that normalization of the FLC level is not associated with better survival rate in patients whose CR meets the traditional criteria. In addition, it was suggested to replace identification of FLC with that of heavy chains, which should be considered more a surrogate marker of immune system recovery than an MRD monitoring item; moreover, FLC testing was criticized as reliable method of assessment of MRD in myeloma, although FLC ratio is one of the response evaluation criteria.

Morphological study

Morphological study of BM is the most common method for determining tumor load in MM cases. Several large-scale studies have shown the independent prognostic value of BM microscopy [16, 17], however, the sensitivity of this method is limited by the number of cells sampled and variability of sampling conditions.

Visualization methods

Multiple myeloma differs from other hematological diseases in the patterns of infiltration of BM with MM cells, which vary depending on the type of the disease and sampling location. Moreover, dilution of BM aspirates with peripheral blood can lead to false negative results. These problems, along with the possible extramedullary (EM) lesions, complicate interpretation of results of all MRD tests relying on BM. Therefore, affirmation of the MRD(–) status may be false. Alternative methods, such as imaging [18, 19], monitoring of clonogenic MM progenitor cells [19, 20] or circulating myeloma tumor cells can give additional information about MRD [2]. Sensitive imaging techniques enable reliable assessment of small EM lesions due to the high frequency of EM recurrences in MM cases. Magnetic resonance imaging (MRI) is the most sensitive non-invasive method of detection of skeletal bone foci, assessment of prevalence and nature of soft tissue lesions, and identification of the type of BM infiltration. Inter alia, MRI is the study indicated in cases of monoclonal gammapathies of undetermined significance (MGUS) and smoldering myeloma, as it detects foci measuring 5 mm and, thus, clarifies progression of the tumor process. However, in the presence of necrosis and inflammation, focal lesions may remain over-intense in both responding and non-responding patients, therefore, an unambiguous CR conclusion based on the results of MRI may be impossible.

While MRI does not allow correctly assessing active foci after myeloma therapy, positron emission tomography (PET) has proven its prognostic significance [18, 21] and may be the most effective method for monitoring MRD in MM cases. The specific advantage of PET is the ability to identify both bone marrow and EM lesions, and to separately show tumor and necrotic tissues. Despite the PET/CT combination being common in clinical practice, it has a number of problems: not all MM patients have detectable foci, and interpretation of data is complicated by heterogeneity of the imaging criteria and insufficient reproducibility in various studies. Moreover, PET/CT is not always sufficiently informative because of spatial resolution limit of 0.5 cm and potential for false negative results when the level of absorption of fluorodeoxyglucose is very low. For repeated examinations, it is necessary to factor in radiation exposure, which is higher than peculiar to radiography and CT [22, 23].

A more specific PET/CT with fluorodeoxyglucose (^{18}F-FDG) is considered a standard imaging method for assessment of efficacy of treatment. Persistence of significant abnormal ^{18}F-FDG uptake after treatment is an independent negative prognostic factor, which substantiates the importance of this MRD diagnostic method when used before starting maintenance therapy. The definition of complete metabolic

response as detected by PET has recently been standardized, and interpretation criteria harmonized. Researchers note promising results shown by innovative radiopharmaceuticals (small molecules targeting CXCR4 chemokine receptors, isotope-labeled CD38 antibodies) as potential theranostics that are both diagnostic and antitumor agents [24].

Allele-specific oligonucleotide PCR (ASO PCR)

A relapse in an MM patient means that not all clonogenic malignant cells were destroyed, and there persist residual tumor cells not detected by the above methods. In this connection, it is important to use more accurate monitoring techniques during remission and relapse, namely, molecular biological methods, including ASO PCR and quantitative real-time PCR. The tumor marker selected in MM cases for MRD assessment is the hypervariable region of rearrangement of immunoglobulin heavy chain genes (IgH). Location of this region and analysis of the sequence require synthesis of allele-specific oligonucleotide primers and probes of specific design [25].

In the context of identification of clonal rearrangements of IgH, ASO PCR allows detecting very small amounts of tumor PCs with sensitivity of 1×10^{-5}. Unlike qualitative or semi-quantitative PCR methods, ASO PCR accurately quantifies MRD. The method involves synthesis of primers complementary to the junctional region of rearranged IgH genes; they are used to learn the depth of response in BM samples taken at various times, which also requires a baseline (taken before treatment) diagnostic sample.

The advantages of PCR methods of MRD diagnosing are their sensitivity, accuracy, reproducibility, low DNA amount requirements, and indispensability in the context of retrospective studies. On the other hand, they are more complex, expensive, take longer and reveal only the initial tumor clone. Nevertheless, detection of tumor markers with the help of PCR is a common practice in clinical testing of patients for early recurrence or tumor contamination of hematopoietic stem cells (HSCs) during autologous transplantation (autoTHSC). Thus, with fully patient-specific primers/probes, ASO PCR is effective in >90% of MM patients; the method allows detection of dynamic changes of MRD during autoTHSC, regardless of the CR established by traditional accepted methods [26].

NGS

NGS is another technique used to establish the MRD status in cases of malignant lymphoid neoplasia. It is a quantitative method based on the use of consensus primers for universal amplification with sequencing of all rearranged segments of Ig genes found in the clonal myeloma cells [5, 27]. NGS is applicable in more than 90% of cases; its sensitivity is $\leq 10^{-6}$. Utilizing automated data analysis and requiring no expert interpretation relying on knowledge of the tumor clone's characteristics, this method can be used in most laboratories. Moreover, such molecular studies are not affected by genetic heterogeneity and changes in the clonality of malignant cells occurring during treatment. The results of NGS can also be interpreted with the aim to identify subclones and clonal evolution at the MRD stage [4]. However, applicability of this test in the context of stratification of patients into risk groups requires additional validation.

MFC

Currently, MFC is one of the main methods for diagnosing malignant neoplasms, detecting their PCs in BM by aberrant expression of surface markers in approximately 90% of patients. The sensitivity of 6-color MFC is 1×10^{-4} myeloma cells; 8 and more colors, or markers, increase it up to 1×10^{-6} tumor cells, and make the test more specific. The method can also differentiate the expression of light k or λ chains of Ig (IgL) [28, 29]. In recent years, the sensitivity of MFC has increased to $\geq 10^{-5}$ thanks to simultaneous assessment of 8 or more markers in one tube, which allows identifying aberrant PC phenotypes while assessing MRD and counting the sufficient number of cells ($\geq 5 \times 10^6$) [30–32]. Invention of flow cytofluorometry that can detect up to 30 markers simultaneously increased the number of fluorochromes that can be used in one tube, as well as the number of cells examined.

MFC also allows evaluating the role of tumor microenvironment in plasma cell diseases [33] and identifying the possible therapeutic targets on malignant PCs [34].

There have been described many surface markers signaling difference between tumor PCs from normal ones. The most common are CD138, CD38, CD45, CD56, CD19, and cytoplasmic κ and λ Ig light chains. Additional diagnostic markers, many of which are characterized by aberrant expression on the PC, are CD20, CD27, CD28, CD81, CD117 and CD200 [35]. In the context of monoclonal antibodies therapy against CD38 or CD138, CD54, CD229, CD319 may be useful. However, heterogeneity of the expression of these markers, differences in the number of studied events and analysis strategy complicate interpretation of results of various studies and add contradictions thereto [36].

MFC has known value in prediction of results of autoTHSC. Many researchers note that MFC-confirmed 100th day MRD(–) status of patients after autoTHSC is one of the most important predictors of disease outcome, and it is associated with a statistically significant improvement of the PFS indicator regardless of the cytogenetic characteristics [6, 37, 38].

According to a study, 58% of the patients who underwent autoTHSC and received lenalidomide maintenance therapy for 1 year achieved CR, and 68% of them were MRD(–) according to the results of MFC. At the three-year mark, PFS was 77%, and OS 100%. None of the patients who became MRD(–) had a relapse after 39 months (median value) [35].

However, there are factors that limit efficacy of MFC: quality of BM samples (should be high), no standard MFC protocols and variable sensitivity, contents of the monoclonal antibody panels and level of execution in various laboratories [39]. Moreover, first generation MFC is not as sensitive as ASO PCR and NGS.

Next generation MFC

Considering the many options of execution of MFC test, the unified MRD definition criteria should be established by a consensus [40]. A consortium of EuroFlow and IMWG have developed next generation MFC, or NGF (next generation flow), which is more sensitive, relies on a new design, and allows counting larger number of cells. There was created and validated eight-color antibody panel for MM diagnostics: 1st tube — CD45/CD138/CD38/CD56/β2 microglobulin/CD19/cyIgkappa/cyIglambda, 2nd tube — CD45/CD138/CD38/CD28/CD27/CD19/CD117 [41], with 4 basic markers (CD38, CD138, CD45, CD19) and 8 additional ones for subsequent identification, counting and characterization of tumor PCs. This method allows simultaneous analysis of up to 10^6 cells. Software algorithms have also been developed for automatic identification of clonal PCs (i.e. MRD) in BM samples.

International Myeloma Working Group approved NGF as a reference method for establishment of immunophenotypic CR in MM cases. Its sensitivity is up to 2×10^{-6}, surpassing that of the previous MFC tests (10^{-4}–10^{-5}), but it strongly

Table. Comparison of MRD assessment methods utilizing BM samples [7]

	ASO PCR	MFC	NGS
Applicability	60–70%	About 100%	≥ 90%
Need for baseline sample	Yes, requires synthesis of patient-specific probes	No, tumor PCs can be identified in any sample by their phenotypic differences with normal PCs	Baseline samples are needed for identification of the dominant clone; alternatively, the initial state can be learned from stored samples with tumor cells
Sample requirements	< 10^6 cells	> 5 × 10^6 cells	< 10^6 cells, greater amount increases sensitivity
Sample processing	May be delayed; works with fresh and stored samples	Study within 24–48 hours after sampling	May be delayed; works with fresh and stored samples
Sample quality control	Impossible. Requires additional studies	Immediate, with global analysis of BM cell	Impossible. Requires additional studies
Sensitivity	≥ 1 in 10^5 cells	≥ 1 in 10^5 cells	≥ 1 in 10^5 cells
Additional information about contents of the sample	None	Detailed information on the content of leukocyte populations	Information about the repertoire of Ig B-cell genes in the studied samples
Duration and complexity of execution	Requires synthesis of patient-specific primers/probes; may take several days	Takes a few hours, relies on an automated data processing system	May take several days, requires significant bioinformatics support
Standardization	Completed for other diseases (EuroMRD), can be done for MM	Standardized by EuroFlow	Work in progress
Availability	Widely available, there are about 60 EuroMRD member laboratories that undergo quality control twice a year	Most clinics have flow cytometers (4 or more colors). Many laboratories use EuroFlow protocols and kits.	Limited to one company/platform

depends on the correctness of identification of the pathological immunophenotype, which translates into the need for highly qualified specialists [42].

Next generation flow cytometry was shown to perform better than NGS, although on a small amount of data [40]. In a series of experiments, researchers compared the two methods: they used both to test for MRD samples from MM patients that underwent autoTHSC 3 months ago. The specific protocols were LymphoTrack® (NGS) and EuroFlow (NGF). The experiment has shown high correlation between the methods (r = 0.905), although it was concluded that NGF was the preferred one for the task. Three-year PFS, according to NGS and NGF, was higher in MRD(–) than in MRD(+) patients (NGS: 88.7 vs. 56.6%; NGF: 91.4 vs. 50%; p < 0.001 for both comparisons), which translated into better 3-year OS (NGS: 96.2 vs. 77.3%; NGF: 96.6 vs. 74.9%, p < 0.01 for both comparisons). In the Cox regression, MRD(–) status meant similar results of both NGS and NGF tests, but the latter was the preferred one considering PFS (RR: 0.20, 95% CI: 0.09–0.45, p < 0.001) and OS (RR: 0.21, 95% CI: 0.06–0.75, p = 0.02). These results confirm that sensitivity of MFC can be on par with that of molecular methods [43].

Currently, NGF enables the shift to the new phase of quantification of residual disease, replacing "minimal" with "measurable" in MRD [44].

The use of therapeutic drugs based on CD38 antibodies, such as daratumumab [45], which weaken the expression of CD38 antigen on PCs, gave rise to the need for alternative markers enabling identification of normal or neoplastic PCs. For this purpose, CD269, CD319, CD229 and CD54 markers proved to be informative, as they allowed identifying PCs in more complex samples, including long-stored ones [29]. It should be noted that monoclonal antibody therapy does not have such an effect on the results of NGS.

Comparison of methods

Each of the described MRD assessment methods (based on the PC phenotype and/or genotype) has both advantages and disadvantages that should be taken into account (Table).

There is a study [46] that compares applicability, sensitivity and prognostic significance of ASO PCR and MFC for MRD assessment, which involved 170 MM patients who responded to therapy at least partially [46]. Ultimately, data from only 42% of PCR tests were used, the reasons being lack of detected clonality (18%), sequencing failures (10%), and suboptimal characteristics of the ASO PCR results (30%). The comparison of MRD assessments by PCR and MFC revealed a significant correlation of the results delivered by both methods (r = 0.881). The results of PCR allowed allocating patients with CR into 2 risk groups with different PFS (49 vs 26 months, p = 0.001) and OS (not achieved vs 60 months, p = 0.008). Although less widely applicable than MFC, ASO PCR enables evaluation of the effectiveness of therapy and stratification of MM patients into risk groups [46].

The prognostic capacity of these methods has also been compared in the context of the emerging new approaches to MM therapy and novel drugs [47]. The survival curves produced by both methods were almost identical, with very high MRD assessment prognostic values for both intensively and non-intensively treated patients, which confirms the significance of both methods in prediction of results of the therapy. However, neither method can detect EM relapses in 100% of cases.

Thus, ASO PCR and MFC are reliable methods for monitoring the effectiveness of treatment. They can support accurate predictions of the outcomes for patients who underwent autoTHSC and those who did not. ASO PCR has greater sensitivity, but MFC is more common. MFC should be considered the method of choice for assessment of MRD in MM cases, and molecular methods can be regarded as additional tools until clear demonstration of their comparative advantages [48].

Real time PCR has greater sensitivity compared to MFC, but the latter is simpler and faster, so they can complement each other in MRD testing. A study [49] has shown a significant correlation between MRD assessment with the help of real-time PCR and by CD138 expression.

Results of the RV-MM-EMN-441 study show that in patients who underwent autoTHSC, the value of MRD is lower than in those who received cyclophosphamide + lenalidomide + dexamethasone. The progression of MRD was preceded by clinical manifestations of a relapse with a median of 9 months, and biochemical signs thereof with a median of 4 months. The assessment of MRD by both MFC and real-time PCR allowed allocating patients to a low-risk group and improving characterization of the effect of therapy [50].

An ideal MRD testing method should meet a number of requirements, including: high degree of applicability (usable in most cases), high sensitivity and specificity, good executability, availability, short duration, low sample requirements (low amount thereof, simple transportation), reproducibility, proven clinical significance, and cost-effectiveness. A significant disadvantage of the sequencing-based molecular methods is the need for a baseline sample, which is used to establish tumor-specific sequences. Currently, there are no methods that fully satisfy these ideal criteria, but the NGS and NGF meet most of the given requirements [5, 27, 51].

MRD assessment using peripheral blood

Typically, clonal PCs are localized in BM, but sensitive methods can detect small amounts of them in the peripheral blood of most MM patients. Circulating tumor cells usually mean worse PFS and OS. MFC-enabled test for PCs in peripheral blood returned negative for patients with CR and positive in those who suffered a relapse [52].

Small amounts of tumor cells circulating in peripheral blood can also be detected by molecular genetic methods. Although ASO PCR was shown to give significantly lower MRD values in preipheral blood tests than BM studies, patients that underwent autoTHSC and whose test returned negative, 3 months after the operation had better PFS (median 15 months vs 4 months) and OS (median 52 months vs 17 months) values [53]. Sequencing-enabled monitoring of clonotypic cells in peripheral blood helped detect MM recurrence at its early stage. Results of another study of ASO PCR's capabilities showed that this method allows detection of myeloma cell clones with occurrence of less than one cell per 10^6 leukocytes; all in all, the researchers found myeloma cells in the peripheral blood of 96% of patients [54]. Despite the correlation between MM clone value in parallel studies of BM and peripheral blood samples, none of the patients in the described studies reached complete remission. Several studies investigated DNA of circulating cells, searching for small amounts of residual tumor cells, which enables tracking of individual tumor clones [55, 56].

CONCLUSION

Given the importance of determining the MRD status of MM patients in the context of production of novel drugs, improvement of HSC transplantation programs and therapy in general, it becomes especially important to use the most sensitive and informative methods for detecting residual tumor cells in clinical practice.

The ideal MRD monitoring test should detect pathological plasma cells relying on a sensitive, predictive, non-invasive, standardized, cost-effective and affordable approach. Along with the evolution of immunological approaches, there are many new additional ways being developed that are designed to identify residual tumor cells in bone marrow and beyond.

Imaging techniques, such as PET-CT or MRI, can detect residual disease, including extramedullary foci and foci in bone marrow. Moreover, recent studies show that whole body diffusion-weighted MRI (WB-DWI-MRI) can give a more accurate MRD assessment than PET-CT with FDG [57]. Another important MRD test method is NGS with sequencing of IgH/IgK/IgL loci for the purpose of identification of rearrangements of the Ig gene in MM cells. NGS data can be further interpreted to identify subclones, clonal evolution, and growth of individual clones at the MRD stage. MRD should be part of the array of clinical tests, assessed on bone marrow samples using proven and standardized procedures with a high sensitivity threshold, ideally 10^{-6}; currently, the list of such methods includes NGF and NGS.

Based on the analysis of pros and cons of each MRD assessment method, it can be concluded that in general, by sensitivity, the rating starts with NGS or NGF, followed by MFC, then ASO PCR, and by applicability — MFC or NGF, then NGS, then ASO PCR, since the latter requires diagnostic samples to identify patient-specific sequences of clonotypes [4].

Combining NGF, NGS and PET CT under a complex approach to MRD assessment is a promising trend, since MFC or NGS can assess MRD from the intramedullar perspective, and WB-DWI-MRI or PET-CT — from extramedullar one, which, combined, grants more accuracy to the overall assessment of deep remission [58]. Currently, several laboratory and preclinical studies revolve around new methods, such as matrix laser desorption/ionization mass spectrometry, high-performance liquid chromatography mass spectrometry, detection of circulating extracellular DNA, and RNA sequencing at the single cell level [59, 60]. Inclusion of the new alternative methods in the testing array for MM patients may radically change the assessment of MRD in the future.

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NORMAL AND DISEASE-ASSOCIATED LEVELS OF SPECIFIC IGG AGAINST FOOD ANTIGENS

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Tolerance to food antigens is essential for body's sustainable development under constant antigenic load. Specific IgG against food antigens have been extensively studied in the literature over the recent years. The presence of those associated with various disorders and introduction of elimination diets for certain food products result in good treatment outcomes related not only to the gastrointestinal tract. Investigation of the impact of the long-term IgG-mediated hypersensitivity to food antigens associated with the increased blood-brain barrier permeability is also relevant when studying pathogenesis of the central nervous system disorders. However, identification of specific IgG in the generally healthy people having no history of allergy or inflammation currently provides no clear understanding of their nature and functional significance. Specific IgG are of great interest in terms of predicting the development of functional disorders, remission and treatment of disorders, changes in susceptibility to food antigens at certain age. The results of specific IgG studies are equivocal, which confirms the need to study their structure, epitopes capable of activating autoimmune processes considering the combined effects of medication, environmental conditions and social living conditions. The paper provides the analysis of the currently available research focused on studying specific IgG against food antigens. The data on identification of specific IgG in individuals with various disorders are provided, as well as the gender-related and age-related differences in antibody detection, the relationship between the antibody levels and the rate of food product consumption.

Keywords: food antigens, specific IgG, anergy, tolerance, atopy

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УРОВНИ СПЕЦИФИЧНЫХ IGG К ПИЩЕВЫМ АНТИГЕНАМ В НОРМЕ И ПРИ ПАТОЛОГИИ

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Толерантность к пищевым антигенам — необходимое условие для формирования устойчивого развития организма при постоянной антигенной нагрузке. В последние годы достаточно широко изучены специфичные IgG к пищевым антигенам. Их наличие при различных патологиях, а также введение элиминационных диет к определенным продуктам питания дают хорошие результаты в лечении заболеваний, и не только касающихся желудочно-кишечного тракта. Изучение влияния длительной IgG-опосредованной гиперчувствительности к пищевым антигенам, связанной с повышением проницаемости гематоэнцефалического барьера, актуально и при исследовании патогенеза заболеваний центральной нервной системы. Но выявление специфичных IgG у практически здоровых людей, не имеющих в анамнезе аллергии, воспалительных реакций, на данный момент не дает четкого понимания их природы и функциональной значимости. Специфичные IgG представляют большой интерес с позиции прогнозирования формирования нарушений функционирования организма, ремиссии и лечения заболеваний, изменения восприимчивости к пищевым антигенам в определенном возрасте. Результаты исследований специфичных IgG неоднозначны, что подтверждает необходимость изучения их структуры, эпитопов, способных активировать аутоиммунные процессы, учитывая сочетанное влияние лекарственных препаратов, экологической обстановки и социальных условий жизни. В статье проведен анализ современных исследований по изучению специфичных IgG к пищевым антигенам. Представлены данные о выявлении специфичных IgG при различных патологиях, гендерные и возрастные различия при выявлении данных антител, зависимости их концентрации от частоты употребления пищевых продуктов.

Ключевые слова: пищевые антигены, специфичные IgG, анергия, толерантность, атопия

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Food antigen ingestion associated with the development of the defense mechanisms aimed at ensuring tolerance to these antigens takes shape within a few months after birth. Interaction between food antigens and the immune system in the intestine results in generation of Tregs $CD4^+CD25^+$ specific for food antigens, which is crucial for induction of tolerance to these antigens. Furthermore, the Treg cells have an anti-inflammatory effect due to expression of IL10, TFGβ, as well as to inhibition of the basophil, eosinophil and mast cell activity. Anergy, being an essential mechanism underlying tolerance to food antigen ingestion, helps maintain gomeostasis in the intestine in cases of chronic high antigenic load. It should be borne in mind that the today's food production is often associated with exposure to chemical substances negatively affecting the immune system function by causing disruptions in the tolerance mechanism, breaking barriers and increasing

intestinal permeability to food antigens. The use of medications, such as aspirin and non-steroidal anti-inflammatory drugs, can interfere with the barrier function of the intestinal epithelium and increase its permeability, and the effect is enhanced in case of simultaneous food antigen ingestion [1–4]. Disruption of the intestinal barrier causes inflammation in the intestine and autoimmune disorders [5–11].

According to the data provided by the US Food and Drug Administration, the main foods causing food allergy in 90% of cases include milk, eggs, peanuts, hazelnuts, shellfish, wheat, soybeans, fish and other food products containing these allergens as direct or hidden ingredients [12]. Corn, sesame, meat, celery, lupine, honey, fruit and vegetables also have high allergenic potential [13]. However, the allergic reaction is caused not by the food product itself, but by certain allegens it contains. For example, these are casein and whey proteins for milk [14], ovomucoid, ovotransferrin, conalbumin, lysozymes, ovalbumin, etc. for eggs [15], tropomyosin, arginine kinase, myosin light chain for shellfish [16], parvalbumins, gelatin, enolase, aldolase, tropomyosin, etc. for fish [17]. Processed foods may contain certain hidden allergens, which also induce immune response in the body.

There are several mechanisms underlying crossing the intestinal mucosal barrier by food antigens. These can permeate through the small intestinal epithelium due to passages formed by secretory epithelial cells (SAPs), which allows food antigens to enter the underlying mucous membranes of the small intestine. SAP formation is induced by the IL13 cytokine through the STAT6-independent and CD38-cADPR (cyclic adenosine diphosphate ribose)-sensitive pathway, it requires IL-4Rα expression by the small intestinal epithelium [18]. Another variant is represented by capture and transport of food antigens by goblet cells (GAP) associated with developing tolerance to these antigens by means of maintaining the level of the $CD4^{+}Foxp3^{+}$ regulatory T cells and stimulation of the IL10 anti-inflammatory cytokine secretion by macrophages in the lamina propria [19]. Thus, tolerance to the ingested foreign antigen is formed. Food antigen ingestion also becomes possible when the intestinal epithelium tight junctions are disrupted, which is observed in individuals with inflammatory disorders of the gastrointestinal tract. Furthermore, food allergens and some food emulsifiers can have the same effect, increasing epithelial permeability, transport, and allergic sensitization, causing pro-Th2 cytokine activation, and facilitating permeability to other food allergens [20, 21]. Transcytosis mediated by microfold cells (M cells) is the best-studied mechanism underlying food antigen entry. The M cell function is to transport luminal substances in order to induce IgA and T cell responses in the Peyer's patches and lymphoid follicles. Infections, aging, inflammation can reduce M cell density, thereby increasing the body's susceptibility to infections [22]. Moreover, food antigens can be captured directly by the lamina propria antigen-presenting cells (LP-APC) via elongation of transepithelial dendrites (TEDs) into the intestinal lumen. TEDs are capable of squeezing through epithelial cells to capture bacteria without epithelial barrier disruption [23, 24].

The data on the gender-related differences in the range of identified specific IgG against food antigens are ambiguous, however, the majority of researchers note that elevated levels of such IgG are observed in women. According to the findings, women have higher levels of specific IgG against all foods than men, except for IgG against chicken and corn [25]. Women had much more specific IgG against wheat (74% vs. 25.5% in males), corn (77.3% vs. 22.7%) and kola nut (71.9% vs. 28.1%) [26]. A significant increase in the levels of anti-egg and anti-shrimp IgG was also reported in women [27]. Food intolerance is much more prevalent among women than among men [28, 29], which is probably due to the fact that female sex hormones (estrogens) have a pro-inflammatory effect and increase susceptibility to atopy, while testosterone is a potent inhibitor of histamine that is known to suppress the mast cell degranulation [30, 31]. The findings of studies focused on the age-related features of identification of specific IgG against food antigens are also equivocal. There is evidence that individuals under the age of 40 have higher levels of specific food IgG against gliadin, egg white proteins and barley compared to elderly patients [26]. According to the findings, the levels of anti-shrimp and anti-crab IgG increase with age; the levels of IgG against tomatoes, chicken, pork, and codfish decrease starting from childhood and then slightly increase by the age of 45; the concentrations of IgG against eggs, milk, soybeans, wheat, corn, and rice decrease with age [27].

Serum levels of specific IgG associated with various disorders are rather extensively studied, which can be useful for the diagnosis of adverse food reactions. However, the role of these antibodies in the disease pathogenesis is still poorly understood and the clinical benefits of testing for the antibodies are highly questionable. It has been shown that depression in adolescents is associated with higher detection rate of IgG antibodies to food antigens against the background of elevated histamine, S100b protein, and homocysteine levels. Furthermore, the authors believe that the chronic food antigen-specific IgG-mediated hypersensitivity reaction or chronic food intolerance, not chronic low-grade inflammation, underlies the adolescent depression pathogenesis [32]. The IgG antibodies against rice, tomatoes, egg yolk/white, wheat, and corn are most often identified in individuals with Crohn's disease. In this case introduction of the elimination diet contributes to induction of the long-term remission [33]. The feature of response to food antigens is that some of antigens have the structure homologous to that of the body's tissues; when the intestinal barrier is disrupted, ingestion of such antigens causes the immune response, triggering the autoimmune processes [34, 35]. The following food products show the highest degree of homology to proteins of human tissues: milk, wheat, food proteins rich in glycine, glucans, pectins, shrimp tropomyosin, and pork [36–40]. Similarity of the peptide sequences of the antibody against wheat gliadin (EQVPLVQQ) and antibody against the cerebellar nervous tissue (EDVPLLED) has been found in children with autism, thus, antibodies against both Purkinje cells and gliadin peptides can be produced in such patients, which can be the cause of certain neurological symptoms of autism [41]. Type 1 diabetes mellitus is an organ-specific autoimmune disease, which is linked to the effects of the cow's milk proteins by some researchers [42]. It is also assumed that the produced antibodies against the cow's milk albumin can cross-react with the surface protein specific for β-cells (p69) and, therefore, cause their dysfunction. Furthermore, similarity of the cow's milk proteins to proteins of human tissues is considered to be the cause of such disorders, as uveitis, multiple sclerosis, systemic lupus erythematosus, Crohn's disease [43, 44]. High similarity of human aquaporins and aquaporins found in plant-based foods (soybeans, corn, spinach, tomatoes), as well as inhibitors of serine proteinases (serpins) of legumes (beans, lentils, peas, peanuts, lupine, alfalfa, and clover) has been revealed. Aquaporins are membrane proteins found, inter alia, on the blood-brain barrier astrocytes and involved in maintaining homeostasis and water metabolism, electrical activity, neurotransmission modulation, and excitability. Aquaporins of plant-based foods are very stable and, therefore, are ingested in the unmodified form. These can

trigger autoimmune responses to aquaporins of human tissues, which results in sensory impairment and neuroautoimune inflammatory disorders [45]. Glycines of food proteins of meat, chicken, eggs, fruit, vegetables, grains, cereals, rice, soybeans, etc. show molecular similarity to collagen, keratin, actin, and human ribonucleoprotein, thus, their penetration through the intestinal barrier can trigger auroimmune responses. Hypersensitivity reaction to food antigens of cereals and dairy products has been revealed in children with autism spectrum disorder [46]. Hypersensitivity reaction to casein is reported in individuals with metabolic disorders and insulin resistance [47]. Furthermore, the researchers assume that the IgG-mediated hypersensitivity to the casein and soybean antigens increases the risk of anemia and hypothyroidism [48]. Food antigens can play a role in etiology and symptoms of Hashimoto's thyroiditis, which is associated with the significantly higher levels of IgG antibodies specific for plums; a negative correlation between the combined levels of IgG against coffee, tea and the number of symptoms has also been revealed [49]. Food allergy is associated with the decrease in the intestinal IgA levels, increased allergen absorption and microflora alteration [50]. Specific food IgG against kola nut, yeast, wheat, kidney beans, peas, corn, and egg white proteins are most often found in patients having symptoms of allergy and no laboratory confirmed allergy [14]. The data on the effects of the rate of food product consumption on the levels of specific IgG are equivocal. There are papers showing that food consumption is not correlated to the IgG levels [49]. The authors of other papers point to the direct relationship between food product consumption and the levels of specific IgG [25, 51].

CONCLUSION

Specific IgG against food antigens are revealed in individuals with the gastrointestinal tract diseases, metabolic disorders, neurodegenerative diseases, autoimmune disorders, etc. However, the mechanisms underlying intestinal permeability alteration and abnormal tolerance to food products are poorly understood. Despite the studies focused on introduction of elimination diets and its beneficial effects, the role of specific IgG in the disease pathogenesis is still unclear, and the clinical benefits of testing for such IgG are questionable. Identification of IgG against food antigens has some gender-related and age-related features. Thus, exploring the mechanisms underlying the association of abnormal tolerance to food antigens can provide the basis for the development of the therapy methods during treatment and the methods to predict the risk of disorders.

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LATEX ALLERGY

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Latex, made from *Hevea brasiliensis sap*, is the material used to make many medical products, including catheters, balloons and gloves. Hundreds of allergens from natural rubber latex have been identified, and 15 of them were numbered, from Hev b1 to Hev b15. Natural proteins in rubber cause both asymptomatic sensitization and type I IgE-mediated hypersensitivity. Treatment of latex makes use of chemical antioxidants that can also bring about type IV hypersensitivity reactions. Latex allergy is one of the most common causes of anaphylaxis in the operating room, and its prevalence has been growing since 1980s, together with the popularity of latex gloves. It is a well-known problem among medical professionals, with gloves and inhaled aerosol particles being the sources thereof. This study aimed to review the current scientific research and practical data in this only partially investigated area. In addition, increasing the awareness of doctors and patients minimizes the existing risks of latex allergy.

Keywords: latex allergy, latex, latex anaphylaxis, rubber, type I hypersensitivity

Author contribution: Gulko SV — literature search and article formalization; Babadjanova GYu — management, editing, commenting.

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АЛЛЕРГИЯ НА ЛАТЕКС

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Латекс, получаемый из сока каучукового дерева *Hevea brasiliensis*, используют для изготовления многих медицинских изделий, включая катетеры, баллоны и перчатки. Были идентифицированы сотни аллергенов из натурального каучукового латекса, 15 из которых присвоены официальные номера (от Hev b1 до Hev b15). Природные белки в каучуке связаны как с бессимптомной сенсибилизацией, так и с IgE-опосредованной гиперчувствительностью I типа. При обработке латекса добавляют химические антиоксиданты, которые также могут вызывать реакции гиперчувствительности IV типа. Аллергия на латекс — одна из наиболее частых причин анафилаксии в операционной, и ее распространенность возросла с увеличением использования латексных перчаток начиная с 1980-х гг. Она стала широко известной проблемой среди медицинских работников при ношении перчаток и вдыхании аэрозольных частиц. Цель настоящего обзора — изучение актуальных научных исследований и полученных данных в этой пока еще не до конца изученной области. Кроме этого, повышение информированности врачей и пациентов минимизирует имеющиеся риски появления аллергии на латекс.

Ключевые слова: аллергия на латекс, латекс, латексная анафилаксия, резина, гиперчувствительность I типа

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Polyisoprene, commonly known as natural rubber latex (NRL), is the base of a wide range of commercial products, including medical gloves and aircraft tires. The main source of natural rubber is latex, a juice-like liquid harvested from Hevea brasiliensis (Hev b), a tree growing mainly in Africa and Southeast Asia, especially in Thailand, Indochina, Malaysia, and India [1].

Under the bark of Hevea brasiliensis, there is a network of latex vessels that contains natural rubber, which is a compound of polymer hydrocarbon 1,4-cis-polyisoprene, water, cytoplasmic organelles, and several enzymes and structural proteins involved in biosynthesis of polyisoprene, latex coagulation, and protection of plants from microbes. Some of these proteins are strong allergens that can trigger sensitization and allergic reactions at initial exposure and production of human immunoglobulin E (IgE), provoking a number of allergic reactions, upon subsequent exposure [2, 3].

The purpose of this review is to study scientific papers covering latex and analyze the data on latex allergy.

Terminology

The word "latex" can have several definitions. In the context of this review, it refers to a natural polyisoprene substance, a milky or white liquid. It is produced by the cells of various seed plants, such as milkweed and poppy. This liquid is a source of natural rubber, gutta-percha, chicle, and gutta-balata, widely used in medicine. In addition, the term "latex" may refer to an aqueous emulsion of synthetic polyisoprene, nitrile, neoprene or plastic, products of polymerization. This type of latex is used in production of coatings, adhesives, medical gloves, etc.

Hevea latex allergens

There are about 250 different types of NRL polypeptides, and 60 of them can bind to human IgE antibodies. Fifteen key allergens of those 60 were given official numbers (from Hev b 1 to Hev b 15) by the Committee on International Allergen Nomenclature of

Table. Latex allergens from Hevea brasiliensis

Name	Description	Weight (kDa)	Family	Cross reaction
Hev b 1*	Rubber elongation factor	58/14.6	–	Papain, figs
Hev b 2	Beta 1/3 glucanase	34–36	PR-2	–
Hev b 3*	Prenyl transferase	24–27	–	–
Hev b 4	Microhelix	110/115	–	–
Hev b 5*	Acidic protein	16	–	Kiwi
Hev b 6.01	Hevein preprotein (prohevein)	20	PR-3	Avocado, banana, chestnut
Hev b 6.02*	Hevein protein (mature hevein)	4.7	PR-3	Avocado, banana, chestnut
Hev b 6.03	C-terminal fragment of hevein	15.3	PR-3	Avocado, banana, chestnut
Hev b 7	Patatin homologue (Hev b 7.01/7.02)	43–46	–	Potato (patatin Sol t 1)
Hev b 8	Hevea profilinus	14–14.2	Профилин	Pollen, celery
Hev b 9	Hevea enolase	51	–	Mould
Hev b 10	Mn superoxide dismutase	22–26	–	Mould
Hev b 11	Class I chitinase	33	PR-3	Banana, avocado
Hev b 12	Lipid transfer protein	9.4	PR-14	Peach and other stone fruits
Hev b13	Esterase	42	–	–
Hev b 14	Chitinase, glycosidase hydrolases family 18	30.2	–	–
Hev b 15	Serine protease inhibitor	8	PR-6	Wheat
Hev b CitBP	Citrate-binding protein	27	–	–
Hev b CyP	Cyclophilin-rotamase	18	–	–
Hev b GADPH	Glyceraldehyde-3-phosphate dehydrogenase	37	–	–
Hev b HSP80	Chaperone protein	80	–	–
Hev b IFR	Isoflavone reductase	35	–	–
Hev b PRS	Proteasome subunit	2	–	–
Hev b TRX	Thioredocine oxidoreductase	12	–	–
Hev b UDPGP	Uridine diphosphate-glucose-pyrophosphorylase	52	–	–

Note: pathogenesis-associated protein; Sol t: *solanum tuberosum*. * — "Indicator" proteins, used to assess allergen content in rubber products or as markers of environmental pollution.

the International Union of Immunological Societies (IUIS) [4, 5]. Hevea's 15 allergen proteins have a wide range of applications: rubber biosynthesis, plant protection (from diseases), structure and housekeeping. In addition, there were identified 9 other Hevea proteins that can trigger secretion of IgE antibodies (Table).

The most sensitizing Hevea allergens are Hev b 1, 2, 3, 4, 5, 6.02, 7.01, and 13 [4, 5]. Clinical importance of some of them (Hev b 2and Hev b 13) is still a debated matter, but this discussion is mostly academic in nature, since treatment of latex allergy involves removal of all Hev b allergens from the immediate environment of the patient.

Hevea indicator allergens

The table describes four hevea proteins that can be used as "indicator" allergens in the context of assessment of the content of allergens in rubber products or detection of latex in the environment [6]. Two of these allergens, Hev b 1 (rubber elongation factor) and Hev b 3 (prenyltransferase), are found on the surface of polyisoprene rubber particles; to trigger sensitization, they need to directly contact mucous membrane. Hev b 5 (acidic protein) and Hev b 6.01/6.02 (mature hevein) allergens are soluble, they are part of latex cytosol or serum C. In most cases, these allergens are released by impregnated rubber products, especially latex gloves, and transferred through the aerosol powder used to put on gloves, or pollute the environment. Medical professionals are exposed mainly to the above proteins.

Latex, fruit, and pollen cross sensitization

Polyvalent latex allergy implies a combination of sensitivity to latex and certain fresh fruits and vegetable products. This variety of the condition affects from 30 to 50% of people suffering from latex allergy [7]. The respective allergic reactions can be severe, with up to 50% of such triggered by food being anaphylactic. The food containing allergens associated with latex are bananas, kiwi, avocado, chestnut, papaya, white potato, and tomatoes; the structural homology of the allergens in them is similar to that of Hev b allergens in latex (Table). The main pan-allergen behind cross-reactivity of fruits and latex is a protective protein, class 1 chitinase, which is structurally homological to Hev b 6.01. Hev b 5 is homological with acidic protein of kiwi, peach, and apricot, and Hev b 6.02 — with agglutinin and endochitinase of the wheat germ in avocado and banana. Hev b 7.01 and Hev b 7.02 are esterases structurally homological with patanine patatin (Sol t 1), the main storage protein in potato. Hev b 8 is a profilin promoting cross reactivity with other highly sensitizing profilins of trees, herbs, pollen of weeds, and food [8–10].

Hevea latex treatment

Centrifugation can separate NRL into three layers [1], with the topmost containing natural rubber particles insoluble in water and having a high content of Hev b 1 and 3, the middle layer, or serum C, containing soluble proteins and plant enzymes, including Hev b 5, 7, 8 and 9, and the lower layer — a

precipitate, or serum B, consisting of heveamines, hevein, and other proteins with chitinase and lysozyme activity. This fraction has high content of Hev b 2, 4, 6.01/6.02, 7, 10, 11 and 13. Serum B and C proteins are water-soluble; they are used in production of diagnostic extracts of skin tests.

There are two approaches to treatment of NRL [11]. Approximately 90% of NRL are acid coagulated and used as base for molded rubber products: tires, plungers for syringes, and shoe soles. This process makes the items less allergenic. The remaining 10% are ammoniated and turned into rubber products: gloves, catheters, and balloons. These items have high content of latex allergens, including Hev b 5, Hev b 6 and Hev b 13. They are the key cause of allergic reactions to NRL proteins. Current latex gloves production technology involves treatment with protease, which decreases the levels of extractable latex protein in them, but a certain amount of allergenic proteins remains. Powder free latex gloves usually have the lowest content of allergens because they are washed with chlorine.

Epidemiology

In the mid-to-late 1990s, latex gloves of natural rubber caused a spike of latex allergies among medical professionals who used them. Subsequently, powdered latex gloves were largely refused, which pushed down the number of latex allergy cases among medical staff and patients who had several operations [12]. However, such gloves and other natural rubber products, such as urinary catheters, are still used in some countries, which supports urgency of the latex allergy problem there. Florists, food vendors, and patients, such as those on dialysis, are also at risk of developing allergies [13].

In North America and Europe, there were several factors that caused the latex allergy epidemic. In 1992, the U.S. Occupational Safety and Health Administration (OSHA) issued the Bloodborne Pathogens Standard, which prescribed using protective gloves [12] and also added medical gloves to the list of "universal precautions." Thereafter, the technology was changed to quick processing of latex instead of long storage, which minimized the degree of protein denaturation that naturally occurs during such storage. Thus, the amount of allergenic protein in raw materials and finished medical gloves increased, exacerbating the problem of latex allergy among the medical community [12, 14, 15].

Prevalence in the general population

Prevalence of latex allergy varies depending on the size of the population and techniques used to identify new cases. Skin tests and serological methods are designed to detect Hev b 6.02, the most common allergen in latex extracts [16]. In the mid 1990s, between 3 and 9.5% of the general population had IgE antibodies to NRL. However, as NRL was increasingly removed from the production process, the prevalence of latex sensitization decreased to < 1% by 2006. Clinical allergy is even less common, but the respective indicators disregard patients with non-IgE-mediated allergic contact dermatitis [7].

Prevalence among medical professionals

Latex allergy became a serious health problem in the late 1980s, especially among medical professionals who were exposed to hevea allergens via powdered latex gloves, which means both direct skin contact with them and inhalation of aerosols thereof [17]. By the mid-1990s, the prevalence of sensitization to hevea allergens in the medical community was at 12.1%, but with the introduction of powder-free gloves, it decreased to 4–7%. However, balloons, latex plates, and rubber dam sheets used in dentistry still cause latex allergy [18].

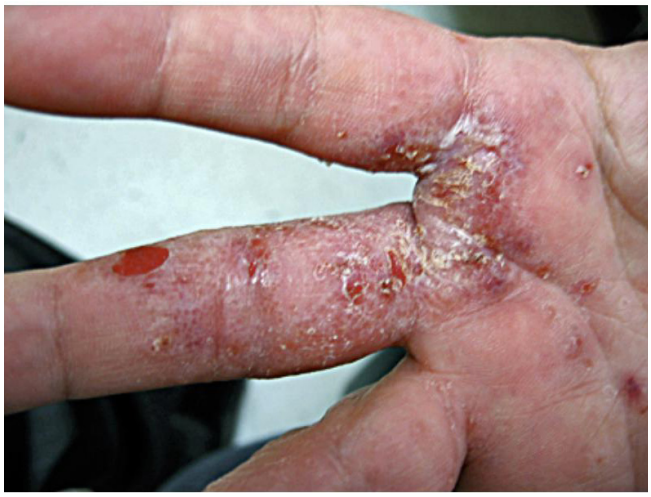

Fig. Contact dermatitis

In Western countries, where natural rubber gloves have been generally abolished, the COVID-19 pandemic weakened state control over the type of gloves ordered. However, in Asia and other regions that have not banned natural rubber gloves on the national level, latex allergy remains an urgent problem [19].

Prevalence of latex allergy among patients who had several operations

Latex sensitization and allergies are common in people who had multiple surgeries, especially on the organs of the abdominal cavity or genitourinary system. Children with spina bifida are considered to be at high risk, as they are often exposed to latex in the context of numerous operations, catheterization of the bladder and manual removal of the rectum. It was estimated that from 1/3 to 2/3 of children who underwent surgery in the 1990s became sensitive to hevea allergens. In some parts of the world, the prevalence of latex allergy in patients with myelomeningocele remains high (19.5%), and more than five surgeries is the most important risk factor for this condition [20].

Risk factors

The main factors that increase the risk of developing latex allergies are professional exposure and atopy. People with eczema or allergies to fruits and vegetables are also more likely to further develop these conditions [21]. Compared to people without atopy, predisposed medical professionals with latex allergies are more likely to have certain polymorphisms of interleukin (IL) promoters, such as IL13 and IL18 [21]. However, in patients with spina bifida or bladder exstrophy and concomitant latex allergy, such polymorphisms were not abnormally frequent. Instead, the risk factors for these patients are the number of previous operations and the history of atopy [22].

Clinical manifestations

The symptoms occurring as part of reaction to latex are shaped by various factors, including method of exposure, amount of allergen present in the natural rubber product, and the main reaction mechanism (irritation, non-IgE-mediated or IgE-mediated) [23].

People wearing medical gloves of hevea latex most often complain of dry, cracked and irritated skin [24]. Erythema

and vesicles are also common. This rash looks like allergic contact dermatitis, but it cannot be attributed to delayed hypersensitivity to additives in gloves. On the contrary, it is an irritant contact dermatitis caused by sweating due to glove occlusion, prolonged contact with alkaline pH medium (made such by corn starch used in many powder gloves), frequent hand washing, and use of aggressive products for this purpose.

Allergic contact dermatitis

Skin rash and itching are common symptoms of allergic contact dermatitis that manifests 1–4 days after contact with a product made of NRL. The rash initially takes form of acute eczematous dermatitis, often with vesicles, then becomes dry, crusted and lichenized. Lichenization (thickening of the skin with emphasized folds or a pattern that looks like deep grooves and wrinkles) is a delayed hypersensitivity (type IVc) mediated by T cells, triggered by oxidizing chemicals and accelerators (thiurams, carbamates, benzothiazoles, thiourea, amines) used in latex production, i.e., it is not a reaction Hev b allergens. However, contact dermatitis may increase the risk of IgE-mediated sensitization to latex due to increased absorption of allergens through skin lesions [25].

Allergic contact urticaria

Allergic contact urticaria or contact dermatitis is an immediate type I hypersensitivity reaction mediated by IgE, manifesting as contact urticaria (Fig.) [26]. This type of reaction is often reported by medical professionals using latex medical gloves. Within 10–15 minutes of exposure, redness, itching, blisters and rashes may appear.

Rhinoconjunctivitis and asthma

In the process of using powdered latex gloves, hevea allergens are released as haze, which can cause symptoms of rhinitis and asthma in people sensitive to latex [23]. Latex-induced sneezing, itching, lacrimation, nasal congestion and runny nose are similar to the symptoms of seasonal pollen allergy.

A history of asthma is not a mandatory prerequisite for development of latex-induced asthma. Allergic symptoms manifesting in the upper and lower respiratory tract can be so severe that some people who are exposed to latex at work have to quit unless their employer totally removes latex from their environment or significantly limits contact therewith [25, 23].

Anaphylaxis

There are reports of anaphylactic reactions to various latex-containing products, both in medical and non-medical settings [25, 27, 28]. The products that most often cause anaphylaxis are:

- gloves;
- balloon catheters;
- dental cofferdams or latex sheets designed to isolate one or more teeth in the oral cavity during treatment;
- condoms;
- bonding glues for hair extensions;
- toy balls;
- pacifiers, teethers, bottle nipples.

Diagnostics

Diagnosing latex allergies can be difficult. The best way to determine if a person is allergic to latex is to carefully study his medical history, especially what concerns exposure and symptoms. Although skin tests, not yet available in Russia, serology and provocative tests can be used to confirm the diagnosis, they have limitations connected with unavailability of reagents, variable sensitivity and specificity, and possibility of severe reactions.

Medical history

Diagnosing a latex allergy requires a thorough clinical history of allergic reactions associated with exposure to products containing NRL [29]. If the patient shows proves hypersensitive to a product (reaction within minutes after contact), and the suspected cause thereof is NRL, it is necessary to investigate all potential allergens, since the first assumption about NRL may be false. For example, there was reported a case of a life-threatening anaphylactic reaction in a woman allergic to cow's milk immediately after using new kickboxing gloves, and it was later discovered that the trigger was not NRL but casein, a component of cow's milk that is part of the glove filler [30].

Latex allergy is associated with various risk factors: hand dermatitis, allergy to fruits/vegetables, and atopy. If clinical history suggests latex allergy, the next step is testing for sensitization to hevea allergens by either epidermic method or search for hevea-specific IgE in serum. Patch tests (application tests) can help differentiate between cell-mediated delayed hypersensitivity reactions to Hev b latex components and immediate hypersensitivity reactions caused by IgE antibodies in response to chemicals added to rubber [29]. Unfortunately, all these tests are not yet available in Russia.

Objective latex allergy studies

In different countries, there are different recommendations for diagnostic tests used to confirm a latex allergy diagnosis.

Study strategies and available reagents

In the USA, the equipment commonly used to detect NRL-specific IgE antibodies in serum are FDA-approved analyzers. The respective systems (ImmunoCAP, Immulite, etc.) are typically operated by clinical immunology laboratories [31, 32]. If the known reagents are available in a country, the first step may be a skin test (injection or puncture), followed by a search for latex-specific IgE antibodies in serum enabled by an automatic analyzer, if results of the skin test contradict the diagnosis based on the patient's medical history [33, 34].

Skin tests

Extracts of whey proteins B and C from NRL are a reliable and safe base for skin tests designed to detect latex allergy. The effectiveness of this procedure can be improved by standardizing allergen extracts and their stability, as recommended in previous studies [29–32].

In Europe and Canada, a skin puncture test usually employs glycerinated latex extracts of hevea from at least three commercial sources [33]. The extracts are prepared with sterile filtered serum C obtained from non-ammoniated or ammoniated NRL; they are glycerinated to keep them stable and prolong their shelf life. Serum C contains both soluble and lutoid allergens released from rubber particles. The non-ammoniated form of serum C, used in European reagents for skin tests, has an extensive allergenic composition.

Diagnosing a latex allergy involves a skin puncture test and successive concentrations of the NRL extract. However,

there have been reports of cases of anaphylaxis caused by this procedure. The sensitivity and specificity of this test ranged from 65 to 96% and from 88 to 94%, respectively, in children with urticaria, rhinoconjunctivitis and/or asthma, whose history suggested latex allergy [34].

In the USA, there are no commercially available reagents for skin tests, and shop-made NRL extracts differ significantly in the content of allergens. Such non-standard extracts undermine trust in the results of the tests, which can be false-positive, and the testing itself can trigger systemic reactions. Puncturing a hevea-containing item is not recommended, since this technique disallows control over the amount of allergen distributed in the skin, thus posing a threat of a systemic allergic reaction as a result of exposure to high doses, or unintentional inhalation [35].

Serology

In the absence of NRL skin test reagents, the preferred alternative is a latex-specific IgE test [29, 34–36]. There are two widely used solutions therefor, ImmunoCAP and Immulite automated analyzers [36]. Noveos analyzer, approved by the U.S. Food and Drug Administration and used in Europe, remedies the problems associated with interference of anti-CCD IgE and exogenous biotin, which may arise with ImmunoCAP and Immulite, respectively. These tests include incubation of human serum with an allergen-containing NRL reagent, and detection of the bound IgE antibody with a reagent labeled by an anti-IgE enzyme. The reported lower quantification limit of these tests is 0.1 kU/l (0.24 ng/ml). ImmunoCAP and Immulite have diagnostic sensitivity and specificity of approximately 70% and > 95%, respectively [37, 38]. A chip-based micromatrix containing eight recombinant Hev b allergens showed better specificity against anti-latex IgE, but it is more expensive and offers analytical sensitivity inferior to that of single IgE assays [39]. ImmunoCAP ISAC can detect latex allergy and sensitization, and identify sensitized but asymptomatic individuals [40]. However, it has only 55% diagnostic sensitivity for IgE antibodies to at least one Hev b allergen, as applied to patients with latex allergy and positive skin tests.

Provocative tests

There are various provocations that aim to induce skin reactions or respiratory allergic symptoms, including glove, nasal, and inhalation tests [41–45]. However, most of these methods are still considered to belong in the realm of research, i.e., they are not recommended for routine clinical practice.

Detection of cross-reactivity food allergies

Patients with latex allergies who specifically request testing for possible cross-reactivity can be prescribed skin prick tests with food extracts or food-specific IgE tests. However, in such situations, skin test or serology without a previous reaction can return a "positive" result confirming secretion of IgE antibodies, which may have no clinical significance and lead to unnecessary measures designed to prevent contact with the allergen.

Mechanisms of development of latex allergy

Latex allergy can manifest as delayed (type IV) or immediate (type I) reactions. Individuals with delayed hypersensitivity triggering contact dermatitis associated with chemical sensitization by accelerants are more likely to develop IgE-mediated systemic reactions (type I) [37]. Thus, everyone with latex sensitivity confirmed by a positive response of IgE antibodies to NRL should be treated the same way.

Latex allergy prevention and treatment strategies

After a confirmed latex allergy diagnosis, there are four applicable prevention and treatment strategies:

- abstention, the most efficient and cost-effective approach implying prevention of contact with NRL allergens [46–50]. In many regions, the prevalence of latex allergy has dropped significantly among healthcare professionals and population in general, and in some cases, it was rendered undetectable by common measures designed to prevent contact with the allergen. This includes a practical latex-safe (not latex-free) strategy adopted by most general and dental clinics, and retirement homes [49];
- pharmacotherapy, which is applicable against acute and chronic allergic symptoms, but it is preferable to prevent reactions and the possibility of increased sensitization. Unfortunately, preventive pharmacotherapy is usually ineffective;
- immunotherapy (IT), which has limited use due to lack of approved therapeutic NRL extracts and high frequency of adverse reactions associated with experimental extracts [47, 50, 51], which have not been approved to this day;

Anti-IgE therapy, which is currently being studied in the context of latex allergy treatment, with no approval for this purpose so far [52]. In some cases, anti-IgE treatment is combined with IT. However, it is important to note that it can be expensive, and its applicability depends on the patient's body weight and the total serum IgE level, which should be in the range from 30 to 700 kU/l [52, 53].

Rejection of latex in clinics, retirement homes, etc.

Latex-safe environment

Creation of completely NRL-free environment is an unrealistic goal. Instead, effective prevention of latex allergies in healthcare settings was realized through creation of a "safe latex environment," which prioritizes control over the effects of latex allergens on healthcare professionals, population, and people allergic to NRL.

Latex advisory committees

Most medical institutions in the United States have established latex committees and programs aimed at eliminating exposure to NRL allergens [48, 54–56]. Interdisciplinary advisory bodies usually comprise local experts in various fields, such as legal, procurement, occupational safety, allergies, and glove use in surgery, anesthesiology, and other branches of medicine [46, 54, 57, 58]. There were also established commissions providing advice on all latex-related issues.

Creation of a latex safe environment includes implementation of policies aimed at replacing NRL-containing products with synthetic alternatives lacking the compounds, or at identifying such products that emit fewer latex allergens. Switch to powder-free latex gloves helps minimize exposure to natural latex allergens in medical settings and other industries where NRL products are often used [59].

Medical/surgical gloves

From 1980 to 2010, powdered examination/surgical gloves were the primary cause of NRL exposure in clinics and hospitals [48, 59, 60]. The amount of allergenic protein released from latex gloves is a measurable indicator, and some institutions

have switched to synthetic alternatives of products with high NRL content [61–63], while others have completely refused gloves containing hevea [49, 54, 56, 58]. Some healthcare establishments created a safer environment by opting for powder-free latex gloves with low allergen content [64, 65].

It may be time to more broadly reconsider the use of NRL gloves that secrete small amounts of latex or no latex at all, along with synthetic medical gloves, which was especially relevant during the COVID-19 pandemic, when they were in high demand. However, currently, there is no generally accepted value that would enable this process, such as < 0.15 mcg of total Hev b 1, 3, 5 and 6.02 per 1 g of a glove, which would be adopted by manufacturers or regulatory authorities and allow describing the respective items as having low allergenic potential, although this issue is being considered [46]. Moreover, is it possible to control the content of total Hev b at every stage of glove production and ensure it never exceeds < 0.15 mcg per 1 g of a glove [66–68]?

Healthcare workers at high risk of latex allergy and sensitized patients

Institutions employing people with latex allergies must follow strict rules to prevent their exposure to the respective allergens. At a minimum, these rules should allow all workers to use powder-free, low protein latex products, and guarantee sensitized people come in contact with latex-free items only. If colleagues of allergic workers use powder-free latex gloves with low protein content, it can alleviate symptoms in them, but not eliminate them completely [69].

Monitoring of NRL products and the environment

Measuring the amount of hevea allergens released from various products, especially medical gloves, and monitoring the levels of these allergens in the workplace air are crucial to confirmation of the properties of new low protein NRL medical gloves in the context of creation of a safe work environment.

ASTM International has approved three standardized tests designed to assess the safety of NRL-containing products and to monitor airborne allergens in workplaces where these products are used. The preferred one is enzyme immunoassay (IEMA; ASTM D7427-08), since it establishes the content of allergens in the product most accurately. At the same time, other tests for hevea allergens, like competitive inhibition [70] based on human anti-latex IgE, are still used in individual laboratories for research purposes, and require large amounts of serum anti-latex IgE [71].

Hev b 1, 3, 5, and 6.02 are the four key allergens monitored in the environment and reflecting the overall level of allergens therein. In food extracts and environmental samples, they can be quantified with the help of IEMA utilizing monoclonal antibodies with two sites (ASTM D7427-08). It is impossible to establish an item's allergenicity by quantifying only Hev b 1 and Hevamine. The results of ELISA of inhibited IgE has shown that a glove can be labeled as having low allergenic potential if the total concentrations of Hev b 1, 2, 5 and 6.02 in it are below 0.15 mcg per 1 g of glove. For a workplace environment, an earlier study suggested a threshold value of 0.5 ng of latex aeroallergens per g/m3 of air. However, this threshold has not been qualified using ASTM D7427-08 IEMA for allergen content [71–77].

Hevea proteins causing antibody reaction can be detected by an enzyme immunoassay of the ASTM D6499 antigen [78, 79]. This method has limitations: it disallows differentiation of latex allergens that induce IgE and antigens that do not induce IgE. Similar to the total protein study, the test for hevea antigen cannot be used to determine if a product or an environment is latex safe, since this label requires an exact assessment of allergen content.

Modified Lowry method was the initial test allowing to measure the total hevea protein content in food extracts or environmental samples (ASTM D5712) [78, 80]. It is one of the colorimetric techniques for determining proteins in a solution, but low analytical sensitivity limit its usefulness in case of allergenic hevea protein. Moreover, this test disallows distinguishing allergenic and non-allergenic hevea proteins. In 2016, ASTM International published information on an immunological method for determination of 4 allergenic hevea proteins, Hev b 1, 3, 5, 6.02. However, this technique allows qualifying the product as containing allergens, but not quantifying the total amount thereof that the product can release.

NRL alternatives

There have been developed synthetic elastomers and hevea-free rubber (Yulex) that are used in production of commercial rubber-like products:

- Synthetic elastomers such as butyl rubber, neoprene (2-chlorobutadiene polymers), and butadiene and acrylonitrile copolymers are commonly used as an alternative to NRL in medical gloves. These materials contain no allergenic proteins and are therefore safer for healthcare professionals and patients with latex allergies. The most common types of non-latex examination gloves are made of nitrile, neoprene, vinyl or synthetic polyisoprene rubber [81].
- In the past, natural rubber from guayula (Parthenium argentatum) was also used as an alternative to NRL [82, 83]. This plant is extremely low in protein, and appears to have no cross-reactivity with NRL allergens either in vitro or in vivo. However, since 2021, the company manufacturing Guayule products has switched from parthenium to low hevea protein latex supplied from Central America, and uses it in production of consumer goods (wetsuits, and, subsequently, medical gloves) [84].

Individual abstention from latex

General approach

Latex can be found in over 40,000 consumer products used in everyday and medical settings, so people allergic to latex should avoid contact with them [84, 85]. In the USA, medical items containing NRL must be labeled thusly.

Duration of contact restriction and possibility of latex allergy reassessment

It is well known that creating a latex safe environment in medical institutions can help alleviate the symptoms caused by latex and hypersensitivity thereto, as reported by staff and patients. However, within 5 years after last contact, latex-specific IgE antibodies can still be detected in the skin and blood of those avoiding exposure to the substance [48, 49, 56, 70, 86–88]. Therefore, the recommendation is to make contact restriction continued.

People with persisting sensitization, running the risk of re-sensitization, can undergo reassessment relying, in the first place, on anti-NRL IgE assays. Therefore, even if subsequent serological tests return negative, it is necessary to take precautions to prevent the effects of latex allergens.

Reassessments are typical before a necessary medical or dental procedure, or during an annual check-up. Anti-latex IgE serology is the only assessment test available in the USA, approved because of the well-documented latex allergosorbent, consistent assay outcomes, and the capacity to give a semi-quantitative result (kUa/L). *In vivo* skin test methods are not available in the USA due to the lack of approved NRL extracts needed therefor. In Europe, patients can choose between serology and puncture skin test, since at least one approved and well-characterized NRL extract is available there. Unfortunately, it is not present in Russia yet.

Additional management issues

Workplace

In the context of monitoring an employee allegedly allergic to NRL, the first step is to confirm the diagnosis using reliable diagnostic methods [46, 57, 64]. In the USA, this is done with the help of several automatic IgE antibodies analyzers approved by the state. In Europe, an alternative thereto is a skin puncture test with an NRL extract. Once latex sensitivity is confirmed, it is necessary to prevent further contact with NRL at the person's workplace.

Although 15 well-described allergenic components of NRL have been thoroughly studied for diagnostic potential, testing for specific IgE antibodies against individual components of the latex allergen does not increase diagnostic sensitivity for latex-induced occupational asthma compared with the detection of IgE antibodies to a natural extract [89]. However, testing for IgE antibodies to latex components can help distinguish different routes of exposure, such as inhalation (Hev b 5/6.02) and mucosal contact (Hev b 1/3).

In Russia, there are two tests available to the patients, a skin allergy test and a blood test. The former involves applying a small amount of latex allergen solution to the person's skin on the forearm or back. Then, the skin is punctured with a needle to let the solution under it. If the person os allergic to latex, there will appear a blister at the site of application of the solution. Therefore, the test is performed by an allergist or a specially trained doctor. The latter, blood test, implies sending a blood sample to a medical laboratory, where it is analyzed (ELISA) with the aim to find allergen-specific IgE to latex (natural rubber). The units of measurement used are IU (international units)/ml.

It is important have documents supporting claims that deterioration of the person's health and disability are the result of latex exposure in the workplace.

Schools

When a student is diagnosed with a confirmed allergy to NRL, systematic treatment thereof begins with the development of an individual health plan and a school-wide prevention plan. It is extremely important to teach the student self-examination skills, especially when there is a risk of anaphylaxis [90].

Following are the measures recommended for prevention of exacerbation and treatment of allergic reactions in people with latex allergies [84, 91]:

- wearing a medical bracelet signaling of a latex allergy;
- prescription of adrenaline for self-administration to patients with a history of systemic reactions to latex;
- use of non-latex gloves;
- announcing the allergy before any medical, dental, gynecological or surgical procedure, as well as requesting a safe environment for people with latex allergies [92].

Immunotherapy

In the context of treatment of IgE-mediated latex allergy, IT is limited by the lack of extracts approved by regulatory authorities, and frequency and severity of adverse reactions thereto.

Conventional subcutaneous immunotherapy (SCIT) utilizing unpurified latex extracts has been tested in several small randomized trials, and shown varying efficacy [93–95]. One study reported alleviation of the symptoms of urticaria and rhinoconjunctivitis, while another showed decreasing respiratory hyperreactivity to latex. However, adverse events, including systemic reactions, often occurred in all studies. In one test, they were frequent both in the introductory and maintenance phases [93].

Sublingual immunotherapy (SLIT) may offer a lower frequency and severity of adverse events than SCIT [96–100], however, the results vary, and, moreover, there were reported cases of anaphylaxis associated therewith [101–104].

Currently, there is ongoing research of the new approaches to IT that seek to reduce the risk of severe adverse reactions while maintaining or increasing efficacy, such approaches employing recombinant allergens, peptides based on the T-cell epitope, and adjuvants that are conjugated or administered with the allergen [84, 105]. These treatments are still experimental.

CONCLUSION

Thus, latex allergy is a set of pathological conditions that combine intolerance to products made of natural or (less often) synthetic rubber with local or systemic reactions that can significantly affect quality of life. This allergy is caused by sensitivity to proteins contained in NRL, and its manifestations vary from skin irritations to anaphylaxis.

It is important to remember that latex allergy can be prevented. People at risk should carefully choose medical and everyday products, and avoid contact with NRL. Many alternatives (synthetic latex or polyurethane products) can be a safe substitute.

Moreover, educating and raising awareness of this problem are key aspects of the latex allergy management. Despite the challenges posed by the condition, preventive measures and proper management of the situation allow most people with this diagnosis to continue living a full and healthy life. Further research and development of new technologies will also contribute to improving the lives of such people.

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COMBINATION OF BACTERIOPHAGES AND ANTIBIOTICS AS THE MOST EFFECTIVE THERAPY AGAINST *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus is a bacterial pathogen that is frequently associated with drug resistance and causes serious infectious diseases. The challenge in treating staphylococcal infections arises not only from the strains resistance to antibacterial drugs but also from the bacteria's capacity to form biofilms. As an alternative to traditional antibiotic therapy, phage therapy, employing virulent bacteriophages, is being explored. Research on bacteriophage's effectiveness against *S. aureus* encompasses both individual use and their combination with antibiotics. The combined approach appears most promising, enhancing therapeutic efficacy substantially through the synergistic action of both the antibiotic and the phage. This review discusses the effects of using both agents together and the methodologies for their evaluation. It summarizes the latest *in vitro* and *in vivo* research on the combined approach against *S. aureus*, including experiments focused on biofilm elimination. Special emphasis is placed on clinical case studies in treating patients.

Keywords: Bacteriophages, *Staphylococcus aureus*, phage therapy, bacteriophage therapy, combination therapy, antibiotics, multidrug resistance, biofilms, synergy between antibiotics and bacteriophages

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КОМБИНАЦИЯ БАКТЕРИОФАГОВ И АНТИБИОТИКОВ КАК НАИБОЛЕЕ ЭФФЕКТИВНЫЙ ПОДХОД БОРЬБЫ СО *STAPHYLOCOCCUS AUREUS*

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Staphylococcus aureus — бактериальный патоген, обладающий способностью к развитию антибиотикорезистентности и вызывающий ряд серьезных инфекций. Проблема терапии стафилококковых инфекций связана не только с устойчивостью штаммов к антибактериальным препаратам, но и со способностью бактерий формировать биопленки. Как альтернатива классической антибиотикотерапии рассматривается фаготерапия — использование вирулентных бактериофагов. Исследования, демонстрирующие действие бактериофагов против *S. aureus*, включают как отдельное использование фагов, так и их комбинацию с антибиотиками. Комбинированный подход представляется наиболее перспективным, так как позволяет значительно повысить эффективность терапии за счет синергического действия антибиотика и фага. В данном обзоре представлено обсуждение эффектов совместного применения двух агентов и методов их оценки. Обобщены результаты последних работ, посвященных комбинированному подходу против *S. aureus* в исследованиях *in vitro* и *in vivo*, а также в экспериментах по элиминации биопленки. Отдельное внимание уделено клиническим случаям лечения пациентов.

Ключевые слова: бактериофаги, *Staphylococcus aureus*, фаговая терапия, бактериофаговая терапия, комбинированная терапия, антибиотики, множественная лекарственная устойчивость, биопленки, синергизм антибиотиков и бактериофагов

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Staphylococcus aureus is a gram-positive microorganism that is one of the main pathogens for human beings causing a wide range of clinical manifestations. This type of bacteria is the main cause of bacteremia and infective endocarditis, bone and joint infections, skin and soft tissue lesions, pleuropulmonary infections and infections associated with use of medical devices. *Staphylococcal infections* are prevalent both in the general population and in hospital settings; their treatment is a challenging task because of the spread of multidrug-resistant (MDR) strains. Previous studies have shown that Staphylococcus aureus ranks second after *E. coli* as a cause of death associated with bacteria insusceptible to antibiotics [1].

Strains of *S. aureus* implement various mechanisms of antibiotic resistance. One of them involves synthesis of beta-lactamase enzymes and production of the Rvp2A protein, an alternative transpeptidase [2, 3]. The latter grants protection from natural and synthetic betalactams; the respective evolution yielded a clinically important group of resistant strains called MRSA (methicillin resistant *Staphylococcus aureus*). Against vancomycin, *S. aureus* can build a thick cell wall that prevents

penetration of the antibiotic [4]. Resistance to aminoglycosides is ensured by rRNA methyltransferase and other enzymes that modify such drugs. Tetracycline-resistant strains often have protective ribosome proteins TetM and TetO [5]. In case of linezolid, *S. aureus* modifies the target sought by this antibiotic, such modification enabled by the spread of mutant variants of the 23S rRNA gene [6]. Efflux pumps play an important role in the development of antibiotic resistance of *Staphylococcus aureus*. Some of them are substrate-specific, like Tet(K) and Tet(L) efflux systems [7]. Others, on the contrary, can recognize and export a wide range of drugs. In *S. aureus*, the latter are membrane proteins from several families: ABC (ATP-binding cassette), MATE (multidrug and toxin extrusion), MFS (major facilitator superfamily), SMR (small multidrug resistance), and RND (resistance-nodulation-cell division) [8]. Moreover, *S. aureus* can build biofilms, cellular aggregates preventing antibiotic molecules from reaching cells. Biofilms also facilitate colonization of various surfaces by *Staphylococcus aureus*, which underpins infections associated with medical devices [9].

In recent years, to effectively treat infections caused by multidrug resistant (MDR) strains, there have been developed alternative approaches, including phage therapy. Bacteriophages (phages) are viruses capable of infecting bacterial cells. Compared to antibiotics, they offer a number of advantages [10]: bacteriophages are highly specific, i.e., there is no risk of disruption of the normal flora nor their self-replication, and they are highly likely to reach the focus of infection; the mechanism of action of bacteriophages, as a rule, is different from that of antibiotics, which makes them effective against antibiotic-resistant strains; another important advantage is the relative simplicity of bacteriophage isolation and subsequent production of the medicines based on them [11].

Despite the potential for bacteriophages to replace conventional antibiotics, several challenges hinder their widespread use in clinical practice. The main barriers have to do with bacteriophage registration and application: the former is a complex and costly process, the latter lacks approved protocols [12]. Other factors that should be mentioned in this context is the bacteria's potential to develop resistance to phages, and their strain specificity, i.e., a narrow range of action [13].

Use of bacteriophages in combination with antibiotics is one of the main ways of their introduction to therapy regimens considered. Currently, many in vitro experiments and clinical studies show efficacy of simultaneous action of these two agents [14, 15]. According to a number of experts, such an approach should significantly simplify registration and patenting of the medicines significantly [16]. Moreover, a combination of two agents with different action patterns can be relevant against MDR strains [11].

This work aims to review the current results of research analyzing treatment of infections caused by *S. aureus* with the help of bacteriophages, alone and in combinations with antibiotics. Below, we look into both *in vitro* and *in vivo* (animal model) studies investigating the effectiveness of phage-antibiotic pairs, and present the results of works experimenting with such pairs as means against biofilms of *S. aureus*, as well as components of complex therapy regimen designed to combat infections caused by the bacteria.

Results of combined use of bacteriophages and antibiotics and methods of their assessment

The efficacy of combination of antibiotics and lytic bacteriophages was first demonstrated in 1941, when the phages were used in combination with sulfonamide preparations against *S. aureus* and *Escherichia coli* [17]. Later, an animal study confirmed positive effects of the combination [18]. Similar results were achieved for the phage and penicillin pair [19]. Combined therapy was successful against infectious diseases like endocarditis, bacteremia, osteomyelitis, and peritonitis [18, 20].

The term "synergism" ("synergistic effect") was introduced much later, only in 2007. A group of researchers has described enlargement of *E. coli* culture lysis zones when targeted by a bacteriophage augmented by sub-inhibitory concentrations of antibiotics (aztreonam, cefotaxime, ticarcillin, piperacillin, ampicillin, nalidixic acid, mitomycin C) [21]. The main explanation for the observed phenomenon was the increased production of bacteriophage particles due to abnormal growth of bacterial cells in the presence of antibiotics. Over time, the term "synergy" has acquired a broader meaning. In particular, the term became applicable to cases when the effectiveness of a phage and antibiotic combination significantly exceeds the sum of their individual effects [15, 16]. Some authors began to introduce additional terminology around positive effects of such combined therapy. For example, in one study, they are divided into an additive effect, synergism, and facilitation, with the first of these understood as resulting in cell growth arrest enabled by the two agents that equals the sum of the effects of each component individually, the second as a stronger version of the first, and the third as the combination having the bacterial growth suppression effect significantly more pronounced that that achievable with the most effective agent alone, but still weaker than the additive effect [15]. The same study also describes the neutral effect of the combined therapy, when a combination's action is as strong as that of its most potent component, and antagonism, when such therapy is less effective than individual use of the agents [15].

The growing interest in combination therapy yielded a variety of laboratory methods designed to assess its effectiveness. In the first works on the subject, the parameter measured was the diameter of plaque size caused by the phage in combination with a sub-inhibitory concentration of an antibiotic [21]. Currently, this traditional approach is still practiced [22]. but the more common methods nowadays aim to measure optical density of the cells infected with antibacterial agents, one of them or both [13, 15]. The suppressive effect is appraised through calculation of the areas under growth curves or by evaluating optical density of the culture after 16–24 hours [13, 15]. This approach is popular because of the clarity and experimental convenience. Colorimetric measurements aimed at estimating the number of living cells (including biofilms) after treatment with antibacterial agents are less common [23, 24]. There was developed an experimental system of continuous cultivation that allows registering pharmacodynamics of the process in addition to revealing the efficacy of combined therapy [25]. A group of researchers has described an isothermal microcalorimetry method for assessing the effects of phages and antibiotics on bacterial biofilm [26]. In the context of in vivo studies employing animal models, the controlled parameters are survival, bacterial load, duration of the infection process, size of the lesion (edema), histopathological indicators, etc. [27–29].

Thus, the increased interest in the joint use of bacteriophages and antibiotics has ushered introduction of the new terms describing the respective effects, and a number of methods were adjusted to the purpose of studying the combined approach.

Combined use of bacteriophages and antibiotics against *S. aureus* in *in vitro* experiments

In *in vitro* experiments, bacteriophages were paired against *S. aureus* with virtually all commercially available

Table 1. *In vitro* studies of the effect of combined bacteriophages and antibiotics on *S. aureus* strains

Year	Phage	Family	Antibiotic	Result	Reference
2012	SA5	*Herelleviridae*	Gentamicin	Synergism	[25]
2018	SA11	*Herelleviridae*	Ampicillin, cefotaxime, kanamycin, tetracycline, ciprofloxacin, mitomycin C, sulfamethoxazole, trimethoprim	Synergism (ampicillin, cefotaxime, tetracycline, ciprofloxacin, mitomycin C, trimethoprim)	[32]
2020	Sb-1	*Herelleviridae*	Daptomycin, vancomycin, ceftaroline, cefazolin	Synergism	[33]
2021	Cocktail AB-SA01	*Herelleviridae*	Vancomycin, ceftaroline, cefazolin	Synergism (vancomycin, cefazolin)	[13]
2021	Henu2	Temperate unclassifiable	Clarithromycin, linezolid, cefotaxime, tetracycline, ciprofloxacin	Synergism	[31]
2021	PYOSa	*Herelleviridae*	Tetracycline, oxacillin, vancomycin, kanamycin, azithromycin, daptomycin, rifampin, linezolid, streptomycin	Antagonism (tetracycline, azithromycin, linezolid, vancomycin, daptomycin, kanamycin)	[34]
2021	Sb-1	*Herelleviridae*	Oxacillin	Synergism, additive effect, facilitation, antagonism	[15]
2022	φSA115, φSA116	*Herelleviridae*	Tetracycline, gentamicin	Antagonism	[22]
2022	vB_SauM-515A1	*Herelleviridae*	Oxacillin, vancomycin, gentamicin, tetracycline, levofloxacin, linezolid	Synergism (tetracycline, linezolid, oxacillin)	[14]
2023	vB_Sau_S90	Temperate unclassifiable	Fosfomycin, ciprofloxacin, vancomycin, oxacillin	Synergism	[35]

antibiotics: aminoglycoside (gentamicin), beta-lactam (oxacillin), glycopeptide (vancomycin), macrolide (clarithromycin), oxazolidinone (linezolid), tetracycline (tetracycline), cephalosporin (ceftaroline, cefazolin), cyclic peptides (daptomycin), etc. (Table 1). As a rule, this approach involves virulent bacteriophages of the Herelleviridae (formerly Myoviridae) and Rountreeviridae (formerly Podoviridae) families, with the former being the preferred option due to their extensive lytic capabilities (they can lyse 80–95% of strains) [30]. In some studies, researchers also use temperate bacteriophages, but only in the context of *in vitro* experiments [31].

As Table 1 shows, bactericidal and bacteriostatic drugs of various classes are included in experiments as antibiotics, and a significant number of studies consider the effect of vancomycin and oxacillin due to their clinical significance. For example, it has been shown that Sb-1 phage (*Herelleviridae* family) and vancomycin, combined, synergistically boost each other against VISA (vancomycin intermediate *S. aureus*) strains [33]. Moreover, the authors have found that use of two antibiotics of different classes (daptomycin or vancomycin with ceftaroline; daptomycin or vancomycin with cefazolin) with a bacteriophage also yields synergy. It should be noted that a trio of a phage and two different antibiotics does not have an effect significantly different from that of a phage-antibiotic pair provided this combination yields synergy. Henu2, a temperate bacteriophage, combined with vancomycin was observed to enhance inhibition of bacterial growth [31]. In a sample of 27 strains, it was shown that Sb-1 phage (*Herelleviridae* family) in combination with different concentrations of oxacillin, in most cases, boosts bacterial growth arrest through synergism, additive effect, and facilitation [15]. The researchers note that cases of antagonism, when phage and antibiotic weaken one another, were extremely rare. Similar results were registered for vB_SauM-515A1, a lytic bacteriophage: combined with oxacillin in certain concentrations, it improved the antibacterial effect, with no cases of antagonism seen in any of the the considered cases [14].

Table 2. Studies dedicated to combined therapy against *S. aureus* biofilms

Year	Phage	Family	Antibiotic	Result	Reference
2011	SAP-26	*Rountreeviridae*	Azithromycin, vancomycin, rifampicin	Synergism (rifampicin)	[23]
2014	MR-5	*Herelleviridae*	Linezolid	Synergism	[41]
2018	SATA-8505	*Herelleviridae*	Cefazolin, vancomycin, dicloxacillin, tetracycline, linezolid	Synergism (vancomycin, cefazolin) Antagonism (vancomycin, cefazolin, dicloxacillin, linezolid, tetracycline) Additive effect (dicloxacillin, cefazolin, tetracycline, linezolid)	[24]
2019	PYO	*Herelleviridae*	Ciprofloxacin, daptomycin, erythromycin, gentamicin, linezolid, oxacillin, tetracycline, vancomycin	Synergism (ciprofloxacin, tetracycline) Antagonism (ciprofloxacin, vancomycin, tetracycline, gentamicin, erythromycin, linezolid)	[16]
2020	Sb-1	*Herelleviridae*	Doxycycline, levofloxacin, linezolid, clindamycin, rifampin	Synergism	[26]
2023	Phage K	*Herelleviridae*	Vancomycin	Synergism	[42]
2023	vB_SauM_Remus	*Herelleviridae*	Vancomycin	Synergism	[43]

Table 3. Clinical cases and *in vivo* studies investigating combined therapy against *S. aureus* infection

Year	Phage	Family	Object	Infection	Antibiotic	Result	Reference
In vivo study							
2013	Sb-1	*Herelleviridae*	Rats	Implant-associated infection	Teicoplanin	Synergism	[46]
2013	MR-10	*Herellevirida*	Mice	Hind paw infections in mice with diabetes	Linezolid	Synergism	[27]
2019	2003, 2002, 3A, and K	Cocktail of phages of various families	Mice	Pneumonia	Teicoplanin	Neutral effect	[28]
2022	vB_SauH_2002, phage 66	*Herelleviridae, Rountreeviridae*	Mice	Endocarditis	Fluoxacillin	Synergism	[29]
2023	vB_SauM_ Remus	*Herelleviridae*	Larvae of Galleria mellonella	–	Vancomycin	Synergism	[43]
Clinical cases							
2019	Cocktail AB-SA01	*Herelleviridae*	–	Infectious endocarditis of a prosthetic valve	Fluoxacillin, ciprofloxacin, rifampicin	Patient recovery	[47]
2019	Cocktail AB-SA01	*Herelleviridae*	–	Infectious endocarditis associated with an auxiliary device in the left ventricle, complicated by sternal osteomyelitis and bacteremia	Cefazolin, minocycline	Patient recovery	[48]
2021	Cocktail AB-SA01	*Herelleviridae*	–	Infection in a prosthetic joint	Cefazolin	Patient recovery	[49]
2022	Mallokai	no data	–	Infection in a prosthetic joint	Daptomycin and ceftaroline	Patient recovery	[45]

The exact mechanisms underpinning the synergistic effect of combined use of phages and antibiotics against *S. aureus* strains are still unclear. Various hypotheses have been proposed to explain this phenomenon. One of them points to the increased production of phage particles in the presence of sublethal concentrations of an antibiotic, as suggested for tetracycline, linezolid, telithromycin, clarithromycin, cefotaxime and ciprofloxacin, which, in the respective experiments, expanded the lysis zones made by the phage, a probable marker of the said increased production of bacteriophage particles [31]. Another study demonstrated sublethal concentrations of antibiotics to cause *S. aureus* cells to swell, which, in some cases, was accompanied by increased production of bacteriophage SA11 (family *Herelleviridae*) [32]. According to the authors, this synergy relies on lysis delay caused by a lack of choline, which is necessary for cell lysis and further release of daughter viral particles. Another explanation for the synergistic effect mentioned antibiotic-induced overcoming of phage resistance, an effect registered for the combination of Sb-1 and vancomycin/daptomycin, which prevented development of resistance to bacteriophages [33]. In addition, an experiment staged in the continuous cultivation system has shown that gentamicin induces formation of cells with a phenotype prone to aggregation into conglomerates, which, in turn, are most sensitive to the phages [25].

Synergism was noted in a significantly greater number of publications than antagonism [15, 22, 34]. Some of them associate the latter with bacteriostatic antibiotics [22, 34], which seems quite reasonable, since bacteriostatic antibiotics are aimed at limiting reproduction and restraining activity of bacterial cells but lack the effect on the protein and nucleic acids biosynthesis systems that triggers death. It is possible, then, that bacteriophages may also be subjected to the said inhibitory effects. Additionally, it should be noted that antibiotics generally reduce the density of bacteria and thus the ability of the phage to replicate.

At the same time, there are noteworthy contradictions in research papers by different authors. On the one hand, some experiments confirm that the ultimate effect a combined phage therapy regimen is strain-specific, and the selection of phage itself is crucial for success [15]. On the other hand, it may be the concentration of the antibiotic that conditions the said effect, its magnitude, or lack thereof. For example, a combination of 10 mkg/ml of linezolid, a bacteriostatic antibiotic, and PYOSa (family *Herelleviridae*) produces an antagonistic effect [34], but at lower concentrations (1–2 mkg/ml) and with Henu2 phages (temperate, unclassifiable), there appears synergy [31], same as in a combination of vB_SauM-515A1 (family *Herelleviridae*) [14].

Thus, combination therapy has significant potential, and in most cases, simultaneous administration of bacteriophages and antibiotics does not reduce efficacy of the agents but has the potential to improve it. At the same time, it is obvious that there are many dimensions to such combinations and their applicability, and the ultimate effect depends on a number of parameters: concentrations of the drugs used, type of the antibiotic, and bacterial strain. A more comprehensive generalization of data requires additional studies investigating correlations between the above aspects, and, for example, factoring in strain typing data.

Combined effect of bacteriophages and antibiotics on *S. aureus* biofilms

Many strains of *S. aureus* can form biofilms, which are increasingly resistant to antimicrobial agents because of their complex spatial structure that mechanically prevents penetration of the antibiotic, and due to the changes in cell phenotype (emergence of slow-growing cells and persistent cells) [36]. Most clinical cases of *S. aureus* infections are associated with biofilms capable of colonization of surfaces of organs and medical devices [37–40].

Combined therapy employing bacteriophages and antibiotics aimed at *S. aureus* biofilms is a subject actively investigated currently (Table 2).

In case of treatment of biofilms, a crucially important factor is the sequence of administration of the agents. Combined

therapy has shown the best results when a bacteriophage is followed by an antibiotic. Presumably, the effectiveness of this approach rests upon the phage's ability to penetrate biofilm matrix and destroy it, which triggers release of planktonic cells and their subsequent destruction by both the phage and the antibiotic [23]. There are many studies that confirmed these findings [16, 24]. Moreover, not only the "phage — antibiotic" sequence (the former of family *Herelleviridae*, the latter vancomycin or cefazolin) was shown to be effective, but also lack of bactericidal results against a biofilm when the considered agents are used separately, and antagonism when the phage followed antibiotics (vancomycin, cefazolin, tetracycline, linezolid) [24]. Another study describes antagonism in cases of simultaneous administration of the agents (vancomycin or tetracycline with bacteriophage PYO (family *Herelleviridae*)), and synergism for most of the tested drugs when they follow the phage [16].

Sequential administration of a phage and an antibiotic was also shown to be effective against biofilms formed by two types of bacteria, *S. aureus* and *Pseudomonas aeruginosa*. For example, a combination of gentamicin (or ciprofloxacin) and a bacteriophage, the former following the latter, completely arrests growth of the biofilm [44]. The authors emphasized that high concentrations of antibiotics (8 MIC (minimum inhibitory concentration)) ensure best results. Classical antibiotic therapy aimed at biofilms also relies on high concentrations of antibiotics. A number of studies have demonstrated the need for such concentrations in combination with bacteriophages when the goal is to eliminate a biofilm [16, 22, 45]. There, concentrations of the antibiotic vary from 2 [16] to 250 MIC [43]. In addition, researchers have shown the dependence of the biofilm elimination effect on concentration of the antibiotic: the degree of biofilm suppression was directly proportional to the concentrations of linezolid and tetracycline and inversely proportional to the concentrations of vancomycin and cefazolin (up to 128 mkg/ml); in the case of other antibiotics (dicloxacillin and tetracycline), no obvious linear dependence was observed [24].

Biofilms are known to play a significant role in implant-associated infections. A group of authors have successfully used a combination of MR-5 (family *Herelleviridae*) and linezolid against biofilms on medical products and devices; they suggested coating orthopedic wires with hydroxypropylmethylcellulose, a polymer carrying mixture of the above agents. The approach not only ensured eradication of biofilms but also weakened adhesion of bacterial cells. In addition, this study showed that two agents used in conjunction decrease the frequency of formation of bacteriophage-resistant mutants [41].

Based on the above, it can be concluded that sequential administration of a bacteriophage and an antibiotic in high concentration ensures elimination of biofilms, and, moreover, a mixture of the two agents can be used together with a polymer coating of medical products and devices. These results can lay the foundation for development of the new approaches to application of implants and catheters.

Studies into combined use of bacteriophages and antibiotics on *S. aureus* infection models; clinical cases

The development of new therapeutic approaches requires confirmation of their effectiveness in animal models. Combinations of bacteriophages and antibiotics are tested on both vertebrates and invertebrates. In former, researchers create models of various infectious diseases, including implant-associated infections, pneumonia, endocarditis, and soft tissue infections induced by diabetes mellitus. Such studies employ the most advanced antibiotics to date, like linezolid, teicoplanin, and vancomycin (Table 3).

Animal studies listed above demonstrate successful application of the combined approach for treatment of infections caused by *S. aureus*. A combination of teicoplanin and Sb-1, a lytic bacteriophage, was shown to destroy biofilms on an intravenous catheter [46]. A study employing a rat model of endocarditis highlighted the prospects of the phage and antibiotic therapy [29]. In an experiment, the most potent combination was that of fluoxacillin and a cocktail of phages of families *Herelleviridae* and *Rountreeviridae*. Another study notes that in animals receiving bacteriophage together with antibiotics, the infectious process is much milder and shorter than in those given only an antibiotic or a bacteriophage [27]. A 2018 work was an exception, however: its authors, using a model of ventilator-associated pneumonia, did not register significant differences between individual use of a phage or an antibiotic and their combined administration [28].

The amount of the reported clinical cases of use of a combination of a phage and an antibiotic against various infections caused by *S. aureus* has been growing recently. A case of 2019, first of its kind, describes successful application of a phage cocktail AB-SA01 (family *Herelleviridae*) in combination with antibiotics (fluoxacillin, ciprofloxacin and rifampicin) to treat prosthetic valve endocarditis [47]. Intravenous administration of the bacteriophage gradually alleviated symptoms (fever, tachycardia, hypotension, and rash) significantly, and lead to a complete recovery. The same bacteriophage preparation was successfully used in conjunction with cefazolin and minocycline in the case of a patient with infectious endocarditis associated with a left ventricular assist device [48]. There was also described a case of successful treatment of an infected joint implant using intravenous infusions of the AB-SA01 phage cocktail and cefazolin, combined with surgical intervention [49]. In all the above mentioned studies, authors noted that bacteriophages are safe, and reported no side effects.

The reports of successful testing of the combined therapy in animal models and positive clinical practice allow a conclusion that use of lytic bacteriophages in conjunction with antibiotics is a promising approach to treatment of *Staphylococcus aureus* infections of varying severity.

CONCLUSION

The use of lytic bacteriophages as an addition to classical antibiotics in the context of treatment of *S. aureus* infections caused by MDR strains has been actively investigated in the recent decades. *In vitro* and *in vivo* experiments demonstrate that frequently, combined administration of a phage and an antibiotic significantly hampers bacterial growth, and the cases of antagonism are much less common. An important advantage of this approach is, undoubtedly, its effectiveness against not only planktonic cells, but also biofilms built by many strains of *Staphylococcus aureus*. Treatment with bacteriophages and antibiotics *in vitro* can resensibilize and significantly increase susceptibility of MDR strains of *S. aureus*. However, the currently available results of *in vitro* and *in vivo* experiments are not exhaustive, and contain many contradictions, which necessitates further research aimed at accumulating and generalizing data. In addition, effective application of the presented approach requires a fundamental basis explaining the mechanisms involved in elimination of *S. aureus* under the combined influence of bacteriophages and antibiotics. Thus, further research should investigate interaction of the phage–antibiotic–bacteria system using methods of systematic biology and omix technologies.

The promising results of application of the combined therapy in patients should be emphasized separately. However, mass introduction thereof requires optimization of the doses of agents and further clinical studies (including a double-blind placebo-controlled study) seeking to confirm the efficacy and safety of using produced properly bacteriophage preparations. Such studies should form the basis for development of the bacteriophages clinical use recommendations.

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ROBOTIC MEANS OF REHABILITATION OF MOTOR ACTIVITY OF PATIENTS IN THE POST-STROKE PERIOD

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Stroke prevalence is one of the most acute problems in the medical and social aspects of society: strokes are the second most common in the mortality statistics of the population. In the Russian Federation, stroke occurs annually in almost 500,000 people and is the first among the causes of death from neurological diseases and the second most common cause of death after heart disease. The most common consequences of stroke are motor disorders of varying severity, manifested as changes in muscle tone, paresis and paralysis, and impaired walking function. This paper is an overview of the current state of robotic rehabilitation devices used for post-stroke limb paresis and of expected trends of their development. The existing variants of their construction, conditions of kinesiotherapy sessions for obtaining the greatest effect are considered. The authors are of the opinion that the nearest prospect for the development of high-tech devices of this type is not only complex stationary universal complexes for clinics, but also simple mobile specialized simulators with remote medical control for outpatient use.

Keywords: medical robotics, devices for rehabilitation, stroke, exoskeleton, biofeedback, functional electrical stimulation

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Author contribution: IY Zemlyakov — article core authoring, formalization of findings and conclusion; DS Zhdanov — analysis of literature; ASh Bureev — analysis of patented solutions; EV Golobokova — search for information on devices for restoration of upper limb functions; YaV Kosteley — search for information on devices for restoration of lower limb functions.

Compliance with the ethical standards: The study was approved by the Ethical Committee of the Multidisciplinary Scientific and Clinical Center for Medical and Sports Rehabilitation and Resorts (minutes №1 dated July 6, 2022).

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РОБОТОТЕХНИЧЕСКИЕ СРЕДСТВА РЕАБИЛИТАЦИИ ДВИГАТЕЛЬНОЙ АКТИВНОСТИ ПАЦИЕНТОВ В ПОСТИНСУЛЬТНОМ ПЕРИОДЕ

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Проблема распространенности инсультов одна из самых острых в медицинской и социальной составляющей жизни общества — инсульты занимают второе место по распространенности в статистике смертности населения. В Российской Федерации инсульт наблюдается ежегодно почти у 500 000 человек и является первым среди причин смерти от неврологических заболеваний и вторым по частоте в структуре смертности после заболеваний сердца. Наиболее частые последствия инсульта — двигательные нарушения различной степени выраженности, проявляющиеся в виде изменения мышечного тонуса, парезов и параличей, нарушений функции ходьбы. В обзоре представлены результаты анализа текущего состояния и возможных направлений развития роботизированных реабилитационных устройств, используемых при постинсультных парезах конечностей. Рассмотрены существующие варианты их построения, условия проведения кинезиотерапевтических сеансов для получения наибольшего эффекта. Ближайшую перспективу развития высокотехнологических устройств данного типа авторы видят в создании не только сложных стационарных универсальных комплексов для клиник, но и простых мобильных специализированных тренажеров с удаленным врачебным контролем для амбулаторного использования.

Ключевые слова: медицинская робототехника, устройства для реабилитации, инсульт, экзоскелет, биологическая обратная связь, функциональная электростимуляция

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Medical robotics is a complex and very specific field that lies at the intersection of several high-tech areas of science and technology. According to D. Engelberger, titled "The Father of Robotics," "hospitals are the perfect place and the perfect environment for robots to be used" [1]. Nevertheless, robotic systems will not be able to completely replace humans in the near future — so far they can only perform routine and repetitive actions [2, 3].

Robotic devices (RDs) in medicine were first used in 1985 to precisely guide needle movement in brain tissue biopsies using a PUMA 560 arm [2]. In the future, the development of positioning surgical systems has become the main focus of medical robotics. However, remotely controlled manipulators cannot be called robotic devices in the full sense, although they have proven themselves in microsurgery [4].

With the development of microelectronics and general robotics, the implementation of RDs in medicine has expanded significantly [5]. Their implementation in laboratory diagnostics [5], surgery [6], psychiatry and psychology [7], dentistry [8] and other areas has become possible. At the same time, the early introduction of service RDs in hospitals to serve patients with low mobility is of high relevance. By performing routine tasks, they significantly reduce the workload of nurses [9].

There is another area of healthcare where RDs may be in high demand. Globally, about 17 million people suffer from strokes each year, losing some or all of their motor function. Survival rates have trended upward in recent years and will reach 70 million by 2030, placing a significant burden on national health and social care systems [10]. RDs for rehabilitation of this category of patients are designed to solve the problem of restoring the functioning of the affected limbs.

The purpose of the review was to conduct a technical analysis of the existing robotic systems for motor rehabilitation of patients in the post-stroke period, and to describe the expected trends of robotics development. Materials were searched in the National Library of Medicine, Scopus, eLIBRARY, Google Patents, and a number of other scientific and patent-oriented databases.

Trends in the development of rehabilitation RDs

Restoration of motor functions of stroke patients is currently possible with the help of external robotic devices (exoskeletons) and electromechanical devices that conduct forced training of the limb in accordance with the methods of kinesotherapy. Electromechanical RDs were first used at the turn of the 1980-90s [11, 12]. By utilizing the feedback sensors of the RD design during exercises, an attempt was made to ensure that the exoskeleton interacted with the human in the atraumatic and most complete manner possible. Thus, the positive effect of exoskeleton use in neurorehabilitation was first described in 1998 [13]. The authors showed the absence of side effects, good tolerance of the prescribed procedures and a significant effect of manipulations with the injured limb on the process of recovery of motor centers of the cerebral cortex.

Over the next 20 years, the number of publications devoted to poststroke neurorehabilitation with the use of RD grew rapidly. In the Russian-language literature, the issue of neurorehabilitation with the use of RD up to 2018 is reflected in the analytical review [14]. The use of RDs in the domestic clinical practice of neurorehabilitation of that period can be estimated by counting the number of cited articles by Russian authors: only 5 out of 71 articles were cited. Another national review mentions more than 240 models of RDs for restorative care [15]. The authors came across findings saying that to fix in memory a motor act it is necessary to perform the exercise at least 400 times. However, in the absence of an RD, it is difficult to do this without errors.

The authors of one review point to the ever-increasing cost of rehabilitation courses for stroke patients in the recovery and residual periods, as well as the high cost of appropriate equipment [16]. This is related to the process of development and implementation of RDs with the possibility of individual adaptation, including the use of artificial intelligence elements. High cost of such products determines a small number of manufactured products given the significant labor input and expenses to obtain appropriate certificates [17]. The second development trend is that more and more mobile compact devices designed for individual continuous use are appearing on the market [18]. Compared to stationary rehabilitation simulators, they are more demanding in terms of materials used, workmanship and energy consumption, which also affects the cost of production. The market for rehabilitation devices is expected to grow by a third to reach $16.6 billion annually over the five years from 2020 to 2025. At the same time, it should be taken into account that the high-tech devices in question are currently available to less than 50% of those who need it [16].

The high burden on the staff of rehabilitation departments, the significant cost of equipment and the scarce number of specialized clinical centers make it necessary to limit the duration of the rehabilitation therapy cycle to a few weeks. The way out of this situation may be the growth of production and expansion of the range of rehabilitation RDs for home use, which are relatively inexpensive due to their narrow specialization and therefore simplified design. It will make it possible to organize a continuous rehabilitation process under periodic medical supervision and achieve positive results in less time. Unfortunately, the domestic segment of the market for personalized rehabilitation RDs is in its infancy and thus is not broad enough [16].

Neurorehabilitation devices

RDs for neurorehabilitation can be qualified as service robots in the subcategory "robots for patient rehabilitation" [19]. Some experts proposed subdividing them into two subclasses: robots designed to train lost motor function after stroke (therapeutic devices) and robots designed to compensate for lost skills (assistive devices) [20]. The relevance of using both types of RD is explained by the fact that they organically complement each other at different stages of rehabilitation. The workload on medical personnel is reduced due to the saving of time for face-to-face control of the correctness of exercise performance, and there is an economic effect expressed in an increase in the number of supervised patients even though there is a minor increase in the workload on one physician.

Devices designed for neurorehabilitation of limbs and their parts can be divided into three types [21–23]:

1) static orthopedic devices whose primary function is that of limb support. They do not have any actuators. These are various types of splints, lumbars, braces and fixators [24];

2) dynamic orthoses that preserve the mobility of the limb. They can be passive, supportive, or active, with mechanical actuators that train a specific joint [25];

3) robotic exoskeletons that replicate the mechanical properties of the limb and, as a result, better match its anatomy. Despite their cumbersome feel and high cost, these solutions are the most suitable for the tasks of neurorehabilitation and functional prosthetics in conditions of free movement.

Let us consider the latter option as the most universal solution, although so far exoskeletons for medical use have not been identified as a separate category in the domestic system of standards [26]. Exoskeletons involve safe, collaborative work with the patient to enable use and improve residual motor function. Consequently, actuation and control systems must provide a minimum of two modes of operation: position-controlled mode and force-controlled mode. In position-controlled mode, the RD moves along predetermined spatial and temporal trajectories defined by its settings. The force-controlled mode relies on the use of the patient's muscular effort to generate a full range of motion in the RD: this mode is suitable for minor muscle paresis. Position control can be added as an additional loop to correct the correctness of the exercise.

The reduction in rehabilitation time using exoskeletons in kinesiotherapy was first shown in paper [21]. At the same time, no significant differences in the effectiveness of exercises with exoskeletons with and without adaptive control were found [22]. The authors even lean in favor of RDs without adaptive capabilities because of their lower cost, higher reliability, and ease of use and maintenance.

The period of the start of rehabilitation measures and the parameters for robot-assisted gate training (RAGT) depend on many factors [23]. It has been found that the best results can be obtained in the acute period of the disease, with a session lasting 30 minutes, three times a week for four weeks. Six clinical parameters were used to assess the condition, including the Fugl-Meyer Sensomotor Function Assessment Scale, the Berg Balance and Balance Impairment Assessment Scale, the Torso Movement Control and Impairment Assessment Scale, the modified Barthel Index for assessing independence in basic activities of daily living, and the modified Ashworth Muscle Spasticity Scale. This statement was confirmed by the results of electromyogram (EMG) studies of a group of 36 patients. The difference of EMG parameters (frequency of peaks, its duration and area) between the control and experimental groups was reliable [27].

Exoskeletons of the upper limbs are more complex in relation to RDs of the same type for the lower limbs. This is due to the fact that the simple movements of the large joints are supplemented by rotations of the hand, as well as grasping or pinching movements of the fingers [28, 29]. However, robotic devices known to date able to perform finger movements, do not take into account the movement of the wrist, so the devices either hold it stationary, or allow it to make movements only in one plane: to bend and unfold. Functional multifacetedness of the simulation of human hand and finger movements implies a high complexity of the task of controlling the RD, including the use of artificial intelligence elements and methods of detecting the patient's movement intentions, including registration of extensometric and electrophysiological signals of paretic muscles [30].

Devices for restoration of upper limb function

There is still no unified coordinated, functionally and physiologically grounded concept of neurorehabilitation measures of arm and hand mobility using robotic devices despite a sufficient number of RD models focused on restoring upper extremity function [31]. This is caused by the ambiguity of existing approaches to neurorehabilitation of stroke patients and the diversity of clinical conditions, which often have no clear distinctions and are combined [31]. As a result of the described situation, there are now available RDs designed to restore hand function based on EMG with brain-computer interface (BCI) and somatosensory RDs with functional electrical stimulation (BCI-FES) [32].

The rehabilitation process using EMG can be based on the principles described below. No significant difference in the effectiveness of the described methods has been found yet [33]:

1) stimulation of the muscles of the paretic limb with electrostimulator signals that correspond to physiological norms and are stored in an appropriate database: the electromyogram is used to monitor the effects;

2) use of the "mirror" principle, whereby an amplified signal is applied to the paretic limb, which is recorded on the healthy limb when the patient attempts to perform identical movements;

3) use of EMG in a biofeedback circuit (biofeedback), when electromyograms are presented to the patient in the "mirror" mode when the patient attempts to make identical movements with the paretic and healthy hand.

RDs using BCI implement different approaches based on recording electroencephalograms (EEG) of motor cortical areas. The main problem of such RDs is the ambiguity of interpretation of the recorded signal. An algorithm based on the analysis of spatial and temporal characteristics of the EEG in several frequency ranges of the total bandwidth of an electroencephalogram signal appears to be relatively simple and specialized even though it requires substantial computational power [34]. A more universal and faster algorithm for minimizing the energy of the signal of the recognized image, allows to obtain approximate solutions, which in some cases turn out to be more effective [35]. The PSD (power spectral domain) algorithm is based on measuring the power spectral density of a signal consisting of a large number of sinusoids generated by independent sources, as observed in many noise-like signals [36]. The general disadvantages of RDs with neurointerfaces include the current impossibility to isolate weak activation signals of small muscles of the hand and forearm that control individual fingers.

Somatosensory RDs are based on the creation of a biofeedback loop between completed sets of movements and sensations received from the visual, auditory or tactile systems of the body [37]. Audio-visual biofeedback combined with virtual or augmented reality technologies, where patients performed exercises with somatosensory immersion effects, proved to be the most effective. Feedback sensors installed to capture movements record force, speed of movement, or position in space of the arm, hand, and/or fingers. Subsequent studies have proven that multisensory stimulation and mechanical feedback to aid in rehabilitation training significantly shorten the rehabilitation process and have long-lasting effects [38].

An effective means of restoring mobility is BCI-FES, in which stimulating pulses induce muscle activity in parallel with forced movements of the entire limb or some part of it. Thus, through reciprocal relations in the motor centers of the cortex, a stable connection between the external stimulus and the corresponding movement is formed. The effectiveness of the method has been shown to restore mobility of both lower [39, 40], and upper limbs regardless of age and gender [41, 42]. At the same time, the greatest effect was demonstrated in the acute phase of stroke. Being slightly inferior in efficiency to somatosensory RDs, rehabilitation simulators of this type can be simpler, cheaper and more compact due to their narrow specialization aimed at training a limited number of movements.

Devices for restoration of lower limb function

Many authors have noted a significant reduction in neurorehabilitation time in patients with lower limb paresis when using robotic exoskeletons, as well as a more effective recovery

of their functioning [43–46]. Recently, flexible lower limb exoskeletons have begun to proliferate, effectively addressing some of the problems of traditional rigid exoskeletons by providing better simulation of the biomechanics of normal walking, increased stiffness at the joints, lighter weight and a relatively compact control system [43].

According to the findings, the attention of lower limb exoskeleton developers over the past decades has focused on three main areas: materials, manufacturing technology and controls [44]. No fundamental improvements have been made to the mechanical part of the design. Biologically neutral lightweight titanium-based alloys and carbon fiber composite plastics have expectedly come to the fore. This makes it possible to significantly simplify the production technology, replacing stamping under the press by modeling the product in a lightweight mold with heating and subsequent solidifying during polymerization of binding resins. Thus, the manufacturing of the basis for the mechanical part of exoskeletons became feasible to small companies. Also, it became possible to customize exoskeleton parts during the production stage. Control of exoskeleton mechanics is developing rapidly, power consumption becoming much lower and elements are becoming more compact — all this due to the emergence on the market of microcontrollers comparable in performance to desktop computers of the early 2000s, as well as miniaturized stepper motors with high torque.

The introduction of BOS to enhance exoskeleton control capabilities appears to be a positive development. One direction is the development of adaptive control based on motion intention recognition using acceleration sensors and percutaneous EMG sensors [45]. In this case, as the authors rightly point out, the main obstacles become the multiplicity of inconsistent scales and assessments of motor activity in post-stroke patients; this makes it difficult to objectively assess the effectiveness of interventions, the lack of adequate mathematical models linking EMG activity of motor nerves with the corresponding leg movement, especially when walking up and down the stairs, as well as the very nature of EMG signals with impaired muscle coordination after stroke, which requires the use of multilayer neural network models for their recognition. Addressing these challenges will allow for partial automation of the rehabilitation process, primarily in terms of modifying the exoskeleton's effect on gait as progress is made in motor skill recovery. The authors rightly note that the introduction of exoskeletons with adaptive control will not only reduce the burden on the rehabilitation physician by taking over the solution of routine tasks, but will also give a significant economic effect due to the increase in the number of patients per one physician.

At the same time, even the use of simplified robotic actuators that implement the motion of only the hip and knee joints during training already has a positive effect on the restoration of walking biomechanics. When analyzing the results of the effect of such a scheme on the recovery of motor functions, we found a general improvement in motor movements, a decrease in extensor muscle tone and an increase in the duration of the support phase in the step cycle; at the same time, the step cycle itself was reduced from five parts to three. The authors concluded that robotic training with active actuators for the hip and knee joints indirectly promotes changes in kinematic parameters in the ankle joint by bringing pattern parameters closer to some average movement pattern [46].

CONCLUSION

Analyzing the works describing the effect of RDs on functional recovery of limbs of post-stroke patients, one cannot but agree with the position stated in one of the works: most sources describe only ideas, at best preliminary design and testing of prototypes, rather than evaluation of devices already in production or ready for mass production [47]. In addition, despite the social significance and importance of the introduction of medical RDs, so far the bulk of proposals in the domestic market is represented by foreign inventions. It should be noted that their high cost and complexity of service maintenance amid sanctions imposed on Russia require a speedy solution of the problems of development and serial production of domestic devices of similar purpose.

The main conclusion of the presented review is that in order to maintain the continuity of the rehabilitation process and really improve the quality of life of patients, it is necessary to develop not only highly effective robotic complexes available for large clinics and rehabilitation centers, but also relatively simple, inexpensive and readily available RDs for home use. This will make the rehabilitation process truly continuous. An example of this could be relatively simple and inexpensive specialized BCI-FES-type RDs for post-stroke patients, the fabrication and sale of which, in our opinion, would not require large investments.

The use of medical service robots for patients with limited mobility at home is still difficult due to the high cost and the need to create an extensive network of service centers. However, the use of such voice-activated RDs in clinical settings is more than justified, as it can reduce the workload of nurses and automate such routine procedures as dispensing medications or monitoring patients' temperature and blood pressure in the morning.

If we analyze the state and immediate prospects for the development of rehabilitation RDs, we should expect their development in two complementary directions.

On the one hand, the emergence of an increasing number of models of universal stationary complexes, oriented for operation in clinical settings and large rehabilitation centers. Initially, each such complex should have a library of profiles of "standard" training sessions of the general plan with the possibility of expansion and supplementation with new combinations of exercises. A prerequisite for such systems should be the use of multi-loop biofeedback, providing individual adaptation to the capabilities of each patient with elements of self-learning. The individual patient profiles developed during the training sessions should be stored in a digital library and used for follow-up visits. At the same time, the distribution of such profiles is hardly advisable due to their high individuality.

On the other hand, to ensure the continuity of the rehabilitation process, we should expect to see the development of a market for relatively inexpensive specialized, possibly mobile, devices for home use. The cost of such RDs can be reduced in case of their functional specialization, use of simplified technologies and unification of the mechanical part and electromechanical equipment, as well as if we keep the set of exercise profiles at a reasonable minimum. But even in this case, the use of at least one biofeedback, allowing to organize adaptation and self-learning of the RDs, should be considered as a necessary condition. Providing these products with the means of objective control (surface EMG, accelerometry) of motor activity of the affected limbs together with the data transmission channel to a remote server will provide the most complete conditions for full rehabilitation measures.

In conclusion, the authors would like to note that the introduction of robotics in medicine is bound to increase the efficiency of diagnostic, therapeutic, and rehabilitative procedures and improve the long-term survival rate of patients. Widespread robotization of healthcare can create conditions for a fairly rapid transition of medicine to a completely different level of diagnosis and treatment, which was recently considered fantastic.

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ASSESSING REHABILITATION OF CONVALESCENT CHILDREN AFTER INFECTIOUS DISEASES

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The fact that the disease sequelae can limit the development of the growing child's activity is the feature of pediatric medical rehabilitation, that is why there is a need for repeated courses of rehabilitation or habilitation, where each subsequent course is a continuation of the previous one. The specialist's mission is to determine indications for rehabilitation. The paper reports phenomenology and methods to diagnose abnormal activity and participation in convalescent children after infectious diseases in order to set the rehabilitation goals in the International Classification of Functioning, Disability and Health domains (categories). The use of method to estimate activity and participation from the point of view of both child and parent or caregiver is considered. The paper provides information useful for specialists dealing with the issues of rehabilitation of children after infectious diseases.

Keywords: children, rehabilitation, infectious diseases, ICF method, activity and participation

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ОЦЕНКА РЕАБИЛИТАЦИИ ДЕТЕЙ-РЕКОНВАЛЕСЦЕНТОВ ПОСЛЕ ИНФЕКЦИОННЫХ ЗАБОЛЕВАНИЙ

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Особенность медицинской реабилитации детей состоит в том, что последствия заболевания могут ограничивать развитие активностей растущего ребенка, поэтому необходимы повторные курсы реабилитации или абилитации, где каждый последующий курс — это продолжение предыдущего. Задача специалиста — выявить показания к реабилитации. В статье представлены феноменология и способы диагностики нарушений активности и участия у детей-реконвалесцентов после перенесенных инфекционных заболеваний для постановки целей реабилитации в доменах (категориях) Международной классификации функционирования, ограничений жизнедеятельности и здоровья. Рассмотрено применение метода оценки активности и участия как со стороны ребенка, так и со стороны родителя или опекуна. Статья содержит информацию, полезную для специалистов, занимающихся вопросами реабилитации детей после инфекционных заболеваний.

Ключевые слова: дети, реабилитация, инфекционные заболевания, МКФ, активность и участие

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The combination of the child's growth and development with the development of activities and skills on the one hand and disabling condition on the other hand is the feature of pediatric medical rehabilitation. Productive communication with the child and his/her parents is also important for pediatric rehabilitation, since family and closest relatives, physical surroundings, are essential for the child's develpment.

Modern medical rehabilitation has changed significantly and is developing dynamically. Now, as never before, it is important to understand and master the tactical rehabilitation techniques, including in the field of pediatric infectology [1, 2].

ICF value and capabilities

International Classification of Functioning, Disability and Health (ICF) describes functional health as interaction between the individual's physical and mental states (the level of body's structures and functions) and his/her ability to manage daily

activities (activity level), as well as his/her involvement in real-life situations (participation level). It is believed that the individual's functioning and disability, including his/her participation, result from interaction between health and the factors of context, or environmental factors (such as air quality, environment accessibility, relationships with peers, availability of services, etc.), and personal factors (such as age, gender, virtues, lifestyle, etc.) [3]. Thus, the disease course and functioning are considered as interactive and evolving processes that can be affected at each of these levels via understanding and modification of human behavior. A clear and functioning-related rehabilitation goal increases motivation and leads to a significantly better outcome. Today, collaborative goal setting as part of a family-oriented approach is widely promoted in pediatric rehabilitation, and the focus is shifted from the level of body's structures and functions to expansion of children's activity and participation in daily activities [4].

According to ICF, human health is influenced by personal and environmental factors extending beyond anatomy and physiology. Alphanumeric characters are used to designate the ICF domains and further divide each domain into categories, thereby ensuring a comprehensive list of disabilities and providing a standard conceptual basis for classification of the components of health and disability [5]. However, such ICF completeness and extensive encoding structure (ICF can describe any deviation in health status) to some extent limited its use in daily clinical practice [6].

In terms of ICF, participation means involvement in real-life situations and activities [7]. Participation takes place in the environment, where a person lives, works and plays. It is important to remember that it is participation in various life situations that is considered as an end-product of rehabilitation of disabled people of any age. Based on definition of participation provided in ICF, it is necessary to use the complex assessment instruments that can be tailored to the community culture and used to estimate children's participation in various real-life situations.

Advanced instruments for assessment of children's participation

The modern instruments measuring children's participation in meaningful activities are as follows: Children's Assessment of Participation and Enjoyment (CAPE) [8], Pediatric Activity Card Sort (PACS) [9], Children Participation Questionnaire (CPQ) [10], and Life-Habit [11]. The listed above instruments do not cover all speres of activity. For example, the frequently used CAPE scale provides good psychometric characteristics for disabled and non-disabled children, estimates participation in important events in the field of leisure and play. This scale does no allow one to estimate participation in such fields, as daily activities, instrumental activities, daily life and rest/sleep. Thus, there are two options for assessment of children's participation in meaningful activities: using the combination of several scales/instruments (CAPE, PACS, etc.), or using an instrument providing comprehensive assessment of participation in various real-life situations [12].

Participation is a multidimensional construct that is influenced by multiple factors (such as gender, age, performance skills), as well as by environmental factors (such as accessibility, social and economic status). Given the definition of participation provided in ICF and the fact that participation is considered to be the end-product of rehabilitation of disabled people [13], it is important to thoroughly and adequately assess participation in various life aspects using inclusive and complex instruments for goal setting, realization of treatment programs and intervention efficiency estimation [14].

Value of contact with parents for pediatric rehabilitation

In pediatric rehabilitation, a full contact with parents and their involvement in rehabilitation activities, specifically in rehabilitation goal setting, provide the basis for successful work. However, the literature data suggest that physicians, who make an effort to determine the goals of the patient and the family, often set the goals that do not reflect the patient's or caregiver's preferences. Patients often consider goal setting as a kind of implicit agreement between the physician and the patients. Sometimes the family and the patient are not aware of rehabilitation goals [14], while collaborative goal setting allows the patient and his/her family to define their interests and help develop the rehabilitation plan [15]. Collaborative setting of goals and objectives in adults is associated with the increased patient's motivation and better treatment outcomes. As for pediatric population, improving the competence of caregivers during collaborative goal setting turned out to be the resource [4].

The experience of physical medicine and rehabilitation (PM&R) physicians shows that parents often feel uncomfortable when setting goals for children of early age due to the lack of knowledge about the condition and the rehabilitation interventions available [16]. In such cases parents can rely on the physician's expertise to set achievable and meaningful goals. Frankly speaking, this limits the depth of cooperation between patient and the family. In other cases PM&R physicians can feel more comfortable when using simplified goal-setting methods not involving the patient and the caregiver. Practitioners sometimes cast doubt on the ability of patient and the family to set realistic goals [17]. However, according to the literature data, understanding of rehabilitation goals by caregivers is improved when the patient, his/her family and physician set the goals together [18]. Moreover, the data show that caregivers and physicians often have different views on improvement during rehabilitation, which emphasizes the importance of the goal-setting model focused on ICF that guarantees that the goals would remain significant for patient and his/her family [6].

Determining the rehabilitation goal in pediatric practice

Rehabilitation goal is usually determined before the beginning of rehabilitation course. During the meeting rehabilitation specialists ask the patient and his/her family the following questions: "What matters most to you?", "What would you like us to help you achieve?" Among identified ICF domains, 3–5 most important ones are selected. The SMART (Specific, Measurable, Achievable, Relevant, and Time-Bound) goal is set based on these domains.

Limitations of rehabilitation goal setting in the "activity and participation" domains in children over the age of five years can be overcome by using the CASP questionnaire.

The Child and Adolescent Scale of Participation (CASP) measures the children's participation in activities at home, at school and in society relative to children of the same age [19, 20]. The scale was developed as part of the program "Child and Family Follow-up Survey" to monitor the results and needs of children with traumatic and other acquired brain injuries. The content and methods used in CASP that are based on ICF [7] have made possible the studies aimed at assessing participation of children/young adults with various chronic disorders, including disabling ones, as well as the studies

aimed at assessing environmental factors, phactors of physical and social surroundings that support or impede functioning.

Capabilities of CASP scale

Regardless of some limitations, CASP remains a brief and relatively simple to complete instrument offering a good coverage at the level of the "activity and participation" domains. Due to its brevity and simplicity it is useful in clinical practice, as well as for assessment of programs and population studies.

At the same time, CASP is one of very few measures of activity and participation at home, at school and in society for children and young adults with chronic disorders/disability, which can be used for both parents and children.

CASP consists of 20 ordinal-scaled items and four subsections: 1) Home Participation (6 points), 2) Community Participation (4 points), 3) School Participation (5 points), and 4) Home and Community Living Activities (5 points). The 20 items are rated on a four-point scale: "Age Expected" (Full Participation in the subscale), "Somewhat Restricted", "Very Restricted", "Unable" ("Unable" in the subscale). The "Not Applicable" response is selected in cases, when the item describes activity, in which the child must not participate due to age (for example, work).

Most of the items are applicable to children of five years and older, that is why it was suggested to use CASP for children starting from senior preschool age.

Each CASP item considers a broad aspect of activity or real-life situation. The item, subsection and total score can be used in research and practice. Higher scores reflect more active participation in community life in accordance with the age-related expectations. CASP also contains open-ended questions about effective strategies and supports, as well as about the obstacles affecting participation (for CASP protocol see Appendix).

CASP can be used for planning of distinct interventions, estimation of rehabilitation efficiency, and research. CASP does not include demographic data, that is why supplementary demographic information is required (for example, age, gender, disability type, organization, geographic location, time since diagnosis).

CASP has been translated into different languages. About 10 min is required to apply CASP. The specialists using CASP for their purposes must be aware of the content and estimation scales of CASP, the key terms subject to assessment (specifically "participation" and "environmental factors) as defined in ICF [7, 21]. Self-completion of the questionnaire (in person or via email) by both child having an appropriate skill and parent is possible, like interviewing by specialist in person or by telephone.

The analysis of original sources suggests that the CASP version for children's self-reports in promising from the perspective of assessing activity and participation of the child having a history of acute disorder or having a chronic disease/disability. The questionnaire is likely to almost evenly rank activity and participation to justify the use of questioning the child only, when the main interest of rehabilitation goal setting is focused on working with patient, or the use of parent's report, when no child's report is available (for example, due to the adolescent's cognitive limitations), or the parallel use, when it is important to understand the nuances of differences between the parents' and children's points of view [22].

Despite the fact that children know best about their role in activities and participation at home, at school and in society, the differences between the reports provided by parents and children are not likely to show, whether the study has been conducted correctly or incorrectly. These are more likely to reflect each person's ideas about health, functioning, and child's well-being. It is obvious that the points of view of both child and parent are important for selection of rehabilitation intervention or organizational measures. CASP is an interesting and promising specific instrument for estimation of activity and participation of children with various disorders due to the earlier reported by researchers [21, 22] correlations of impairments identified based on the data from the parents' reports with certain disorders. A more thorough investigation of these correlation involving larger samples for each disorder/disability (for example, for the most prevalent infectious diseases constituting up to 90% of the causes of morbidity of children under the age of 14) can be useful for future research [23].

Features of rehabilitation of children with infectious diseases

It is well-known that up to 50% of all disability cases in children are associated with infectious diseases, and infectious diseases account for about 70% of the death rate of children in their first year of life [24–26]. The Russian experts more than once and in great detail raised the issue of arranging medical rehabilitation for children with communicable diseases due to the possibility of developing persistent severe residual effects [27–29].

The global data suggest that the ICF-oriented approaches to arranging and conducting rehabilitation treatment of children with infectious diseases are used. The results show significant heterogeneity of rehabilitation goals and emphasize that the goals should be assessed individually for each child, regardless of health status and such factors, as age or functional independence [4]. Furthermore, the studies that are of some organizational and practical value are based on the use of both rehabilitation diagnosis in terms of ICF domains and supplementary questionnaires.

Thus, the study focused on rehabilitation of children, who survived bacterial meningitis (BM), showed that children often suffered from the quality of life reduction due to disabling sequelae. The authors wanted to assess the health-related quality of life (HRQOL) and the impact of neurological and auditory sequelae in children, who had a history of BM, using the Pediatric Quality of Life Inventory (PedsQL) instrument to reveal the differences in HRQOL between patients and the control group. The findings showed that survivors had significantly lower scores than controls based on the parent-proxy PedsQL reports, which was indicative of lower quality of life (physical health: 82.5 vs. 100, $p = 0.001$; psychosocial health: 80 vs. 90, $p = 0.005$; total score: 82.61 vs. 93, $p = 0.004$), while the children's PedsQL self-reports showed no differences between cases and control. In all classes of the Glasgow Outcome Scale cases were quite different from the control groups in terms of the parent-proxy PedsQL reports with the total score of 84.21 (mild/no disability), 43.54 (moderate disability) and 55.56 (severe disability), while the score of the control group was 91.3 ($p = 0.04$, $p = 0.02$ and $p < 0.001$, respectively). The parents believed that the BM survivors' quality of life decreased regardless of the presence or absence of disability. Follow-up and timely rehabilitation (if necessary) had to be provided to all BM survivors [30].

The study aimed at exploring the patients' beliefs and perceptions with regard to the needs of their children with congenital Zika virus infection using the ICF criteria is extremely interesting [31]. The findings have shown that, despite the fact that parents actually focused on the issues related to motor

ability of their children, their attention was generally focused on the environmental factors. These factors included services, system and policy for prevention and treatment of children and the factors, that could ensure healthy lifestyle, promote physical and psychological well-being, and contribute to the childeren's social status. Furthermore, given the children's early age, rehabilitation goals had to be adjusted later, when the children would be able to express their opinion [32].

The next study is focused on the importance of systemic approach to determination of all factors affecting the effects of rehabilitation, as well as on the impact of time on the natural course of recovery from acute encephalitis [33]. The study involved the use of five functional outcome measures for patients with neurological impairment or disability, including the Functional Independence Measure for Children (WeeFIM), Glasgow Outcome Scale-Extended (GOS-E), Modified Rankin Scale, International Classification of Functioning (ICF), and Liverpool assessment scale. The WeeFIM components obtained during patient assessment included estimates of assistance needed for moving, daily activities, bladder and gut control, need for transportation means, communicative and cognitive abilities. The clinically significant ICF domains included the degree of difficulty in moving body in space, maintaining sitting position, amount of sleep, maintaining sleep, adequacy of sleep, muscle tone of the whole body, involuntary jerking of muscles, and generalized pain.

ICF core sets for pediatric rehabilitation

It is well-known that ICF includes 1685 categories, which makes reliable goal selection during clinical work very difficult. The ICF core sets (i.e. the short list of ICF categories considered to be the most suitable for an individual with certain health condition) mitigated this problem to some extent. The core sets are developed during the research process involving researchers, physicians, caregivers or patients from all over the world. Currently, there are only three ICF core sets for children and young adults with childhood-onset disability. The core sets have been developed for cerebral palsy (CP) [34]; autism spectrum disorder (ASD) [35]; and attention deficit hyperactivity disorder (ADHD) [36]. The common short datasets represent an international minimum standard for assessment and description of functioning at any age using the lowest possible number of categories [37]. Despite the fact the the core sets have reduced the number of ICF categories per diagnosis, problems with clinical realization persist. For example, the ICF core set for ADHD includes 111 categories, while a common short set for ADHD uses 73–81 categories depending on the age range [36].

CONCLUSION

Using the data of the CASP questionnaire to assess the child's activity and participation (child and parent version) will make it possible to considerably simplify establishing the rehabilitation diagnosis based on ICF, as well as the processes of goal setting and rehabilitation intervention efficiency assessment. Extensive use of ICF-based universal information by multidisciplinary rehabilitation teams, involvement of family members and children with infectious diseases into goal setting, development and realization of rehabilitation plan will contribute to achieving the optimal level of participation in the home, school and social life. In this case the CASP questionnaire is a novel, rather simple and effective instrument to meet these challenges.

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METHYLATION OF CELL CYCLE AND APOPTOSIS GENES' PROMOTERS IN EXPOSED INDIVIDUALS WITH SUBSEQUENT MALIGNANT NEOPLASMS

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DNA methylation plays an important role in carcinogenesis; there are many studies that investigate the degree of methylation of the entire genome, gene promoters, and non-coding elements in cancer cells, but much less information about changes of the methylation patterns in blood cells and links with the development of malignant neoplasms (MN). This study aimed to investigate the degree of methylation of promoter regions of cell cycle control and apoptosis genes (*BAX*, *MDM2*, *TP53*, *NFkB1*) in peripheral blood cells of persons chronically exposed to radiation with MN developing latently. The study included 200 persons chronically exposed to radiation from the Techa River, contaminated with nuclear wastes dumped into it. The level of methylation was assessed by real-time PCR. The participants were divided into exposed and control groups; comparing them, we found that in the former, the distribution of exposed individuals with latent MN by the degree of methylation of promoter regions of *BAX*, *MDM2* and *NFkB1* genes was significantly different from that in the latter ($p < 0.001$; $p < 0.001$; $p = 0.004$, respectively). It was established that, compared to the control group, the share of the test group participants with subsequent MN who had up to 10% of the *BAX* gene promoter regions methylated was significantly higher, and amounted to 98%, while in the control group this figure did not exceed 73% ($p < 0.00001$).

Keywords: chronic radiation exposure, gene methylation, CpG dinucleotides, carcinogenesis, the Techa River

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МЕТИЛИРОВАНИЕ ПРОМОТОРОВ ГЕНОВ КЛЕТОЧНОГО ЦИКЛА И АПОПТОЗА У ОБЛУЧЕННЫХ ЛИЦ, ВПОСЛЕДСТВИИ ЗАБОЛЕВШИХ ЗЛОКАЧЕСТВЕННЫМИ НОВООБРАЗОВАНИЯМИ

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Метилирование ДНК играет важную роль в канцерогенезе, в литературе встречается достаточно много исследований уровня метилирования всего генома, промоторов генов и некодирующих элементов в раковых клетках. При этом данных об изменении паттерна метилирования в клетках крови и связи с развитием злокачественных новообразований (ЗНО) существенно меньше. Цель работы — исследование уровня метилирования промоторных регионов генов контроля клеточного цикла и апоптоза (*BAX*, *MDM2*, *TP53*, *NFkB1*) в клетках периферической крови лиц, подвергшихся хроническому радиационному воздействию в латентном периоде развития злокачественных новообразований. Исследование проводили у 200 человек, подвергшихся аварийному хроническому радиационному воздействию в результате сбросов радиоактивных отходов в реку Течу. Уровень метилирования оценивали методом ПЦР в реальном времени. Было установлено, что распределение облученных лица с ЗНО в латентном периоде по уровню метилирования промоторных регионов генов *BAX*, *MDM2* и *NFkB1* статистически значимо отличалось от распределения в группы сравнения ($p < 0{,}001$; $p < 0{,}001$; $p = 0{,}004$ соответственно). Установлено, что в группе облученных лиц, которые впоследствии заболели ЗНО, доля лиц с уровнем метилирования до 10% промоторной области гена *BAX* была статистически значимо больше и составила 98% относительно группы сравнения, в которой доля таких людей не превышала 73% ($p < 0{,}00001$).

Ключевые слова: хроническое радиационное воздействие, метилирование генов, CpG-динуклеотиды, канцерогенез, река Теча

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To date, the potential usefulness of genetic factors in prediction of risks of malignant neoplasms (MN) has been investigated fairly well. For some MN, there were established highly reliable genetic markers, like *BRCA1* and *BRCA2* mutations for breast cancer and ovarian cancer [1], *TP53* mutations for breast, lung, stomach and intestinal cancers [2], *ATM* gene mutations for pancreatic and breast cancers [3]. However, polygenic nature of MN prevents determination of the role of such changes in radiation-induced carcinogenesis. Epigenetic indicators, including DNA methylation, which are modifiable by environmental factors like ionizing radiation, can underpin an alternative approach to the MN risk prediction.

Epigenetic modifications, including methylation, affect the expression of genes involved in carcinogenesis at different stages, from initiation to progression [4]. Hypermethylation of suppressor genes, mobile genetic elements, and oncogenes, is registered in tumor cells, the examples thereof including hypermethylation of tumor suppressor genes in non-small cell lung cancer, colorectal cancer, breast, prostate, and bladder cancer cases [5–7]. Hypomethylation of mobile genetic elements, such as Alu and *LINE-1,* as well as individual gene regions, was registered in breast, ovarian, hepatocellular, and stomach cancer cases [8, 9].

It should be noted that epigenetic marks reflect both the innate genetic background and the impact of environmental factors, which is important in the context of investigation of the effects exogenous factors have on carcinogenesis [10].

DNA methylation is tissue-specific, therefore, methylation patterns obtained from, for example, blood, cannot be easily extrapolated to tissues in which cancer grows [11]. However, this is possible, since the correspondence between DNA methylation in different tissues depends on the locus and the degree of inter-tissue correlation, and methyl marks can be inherited or form at early stages of development, as a consequence of which they will be detected in many tissues [12]. Changes of methylation patterns peculiar to the aging genes (epigenetic clock) may also be associated with the risk of development of various pathologies, including cancer [13–15].

There are published papers that report development of the MN risk prediction algorithms based on the analysis of blood cell DNA methylation. The phenotypic aging and mortality risk assessment algorithms based on the level of methylation of CpG-dinucleotides of DNA associated with age, plasma protein levels, smoking status, and key disease factors, were shown usable in the context of both overall and specific MN risk evaluation, including that for lung, prostate, breast, colorectal cancers [16-18]. A systematic review of studies investigating human blood DNA methylation established a stable relationship between breast cancer risk and global hypomethylation of blood cell DNA and epigenetic age [19].

However, despite the mentioned works, there is still no reliable evidence of the alleged link between DNA methylation patterns and MN development risks.

Cell cycle arrest and apoptosis are some of the mechanisms preventing cell's oncotransformation; with this in mind, we conducted this study seeking to assess the level of methylation of promoter regions of cell cycle control and apoptosis genes (*BAX*, *MDM2*, *TP53*, *NFkB1*) in blood sampled from individuals who were chronically exposed to radiation and subsequently had MN.

METHODS

Characteristics of the examined individuals

We determined the degree of methylation of CpG dinucleotides in promoter regions of peripheral blood *BAX*, *MDM2*, *TP53*, and *NFkB1* genes in people exposed to chronic low dose rate radiation emitted by the Techa River contaminated with liquid radioactive wastes dumped from the Mayak Production Association in 1950-1960. Individual doses accumulated by red bone marrow (RBM) were calculated for each participant using the Techa River Dosimetry System (TRDS) 2016 [20]. They were divided into two groups: a test group, which included 100 exposed persons who were subsequently diagnosed with MN (we collected blood samples prospectively, in the latent period, 5 years before MN developed), and a control group, which consisted of 100 exposed persons not diagnosed with cancer. In this study, the latent period was up to 5 years, because the level of methylation depends on various environmental factors and may change over time, consequently, a longer follow-up period would weaken the link with cancer risk. One of the previously published systematic reviews has shown that the DNA methylation patterns can change in different periods of observation [19].

The inclusion criteria were: residence in one of the 41 Techa riverside villages from 01.01.1950 to 31.12.1960; availability of the individual red bone marrow dose data calculated based on TRDS 2016 [20]. The exclusion criteria were: autoimmune diseases, hemoblastoses and malignant neoplasms at the time of blood sampling (including in 2023 for the control group).

The following MN were diagnosed in the test group 2002 to 2020: lip cancer (ICD 10 code C00 — 3 cases), cancer of digestive organs (esophagus, C15 — 1 case; stomach, C16 — 14 cases; transverse colon, C18.4 — 5 cases; rectosigmoid junction, C19 — 3 cases; pancreas, C25.9 — 8 cases), cancer of respiratory and thoracic organs (trachea, bronchus, lung, C34 — 19 cases), breast cancer (C50 — 16 cases), cancer of female genitalia (cervix, C53 — 7 cases; uterine body, C54 — 4 cases; ovary and uterine appendages, C56 — 3 cases), male genitalia (prostate gland, C61 — 8 cases); urinary tract (bladder, C67 — 6 cases; kidneys, C64 - 3 cases).

Table 1 presents characteristics of the examined individuals.

The mean age of the examined persons with MN was 68.3 ± 0.7 years (from 51 through 86 years). More than half (54%) of members of this group were female. The average accumulated RBM dose there was 731.5 ± 68.3 mGy (dose range: 10.1–3,507 mGy).

By each of the studied genes, the number of people in test and control groups differed, but by age at the time of examination, sex and RBM dose, the groups were comparable (Table 2).

All participants of the study signed a voluntary informed consent form approved by the Ethics Committee of the Urals Research Center for Radiation Medicine.

Research methods

Genomic DNA isolated from frozen blood samples was denatured and converted with bisulfite using the EpiJET Bisulfite Conversion Kit (Thermo Scientific; USA), as per the manufacturer's protocol. After bisulfite treatment, we applied primers specific to the methylated DNA sites for amplification purposes. Methyl Primer Express Software V.1.0 (Applied Biosystems; USA) was used to construct the sequences of primers for PCR fragments of promoter regions of *BAX*, *MDM2*, *TP53*, *NFkB1*. We selected genes based on the results of earlier studies investigating their transcriptional activity and level of methylation of the gene's promoter regions in irradiated individuals [21, 22].

The oligonucleotides were synthesized by DNK Synthes (Russia). Table 2 shows sequences of oligonucleotides specific to the methylated DNA sequence.

Table 1. Characteristics of the studied groups

Group characteristics		Test group (patients with latent MN)	Control group			
			BAX	*MDM2*	*TP53*	*NFkB1*
Number of participants		*n* = 100	*n* = 73	*n* = 140	*n* = 69	*n* = 90
Age at the time of examination, years: M ± SE (min–max)		68.3 ± 0.7 (51–86)	71.7 ± 0.8 (59 – 87)	71.8 ± 0.5 (56–87)	70.4 ± 0.8 (58 – 84)	71.5 ± 0.7 (59–84)
Sex, person (%)	Male	46 (46)	26 (36)	51 (36)	17 (25)	29 (32)
	Female	54 (54)	47 (64)	89 (64)	52 (75)	61 (68)
Accumulated RBM dose, mGy, M ± SE (min-max)		722.5 ± 69.3 (10.1–3507.1)	542.4 ± 63.4 (10.1–2869.8)	617.6 ± 52.2 (10.1–3179.7)	507.6 ± 62.0 (10.0–2869.8)	765.8 ± 83.3 (10.1–3715.5)

Note: RBM — red bone marrow, M — mean; SE — standard error; *n* — number of people; (min–max) — range of values.

The status of methylation of the gene promoters was established with the help of real-time PCR and high resolution melt curve analysis (HRM analysis). The reaction was triggered in 20 µl of a 5x qPCRmix-HS (Eurogen; Russia) reaction mixture consisting of a highly recessive Taq-DNA polymerase with specific monoclonal antibodies, SYBR Green I dye, a mixture of dNTP, $Mg2^{+}$ and PCR buffer. For real-time PCR, we used a StepOnePlus Real-Time PCR System (Thermo Scientific; USA) amplifier. The temperature and time sequences for this procedure were as follows: first denaturation (95°, 5 minutes), denaturation (95°, 30 seconds), annealing (see Table 2 for annealing temperature for each gene, 30 seconds), and elongation (72°, 30 seconds) — 50 cycles; construction of the melting curve (95°, 10 seconds; 60°, 1 minute; 95°, 15 seconds; 60°, 15 seconds).

Bisulfite-converted samples of commercially available fully methylated DNA, CpG Methylated Human Genomic DNA (Thermo Fisher Scientific; USA), and unmethylated Human Genomic DNA Male (Promega; USA) were used as controls enabling assessment of methylation of the studied CpG islands of the gene promoter regions. The controls were mixed in the following ratios: 0/100, 5/95, 10/90, 25/75, 50/50, 75/25 and 100/0, respectively. The degrees of methylation for each control sample were 0%, 5%, 10%, 25%, 50%, 75% and 100%. For the analysis, we used HRM software (Applied Biosystems; USA); it was based on the comparison of the experimental DNA samples melt curve profiles with the standard samples, i.e., those with a known level of methylation. Based on the standard samples, the following degrees of methylation were distinguished: 0%; 0–5%; 5–10%; 10–25%; 25–50%; 50–75%; 75–100%. Experimental DNA samples were distributed accordingly.

Statistical analysis of the data

SPSS Statistics 17.0 software package was used for statistical processing of the results. Yates's chi-squared test enabled comparison of distribution of the participants by the level of methylation; the differences were considered significant at $p \leq 0.01$. To distinguish between methylation levels of 0 through 10% and over 10%, we used Fisher's exact test. The differences were considered significant at $p \leq 0.05$. Spearman's rank correlation coefficient (R) enabled correlation analysis designed to evaluate the effect of RBM dose and age on the degree of methylation; correlations were considered statistically significant at $p \leq 0.05$.

RESULTS

We found that by the degree of methylation of promoter regions of *BAX*, *MDM2* and *NFkB1*, test group (exposed individuals with latent MN) differed significantly from the control group (see Figure). It should be noted that in the vast majority of those who eventually developed MN, the level of methylation of the mentioned promoter regions did not exceed 10%, and the bulk of differences in distribution as compared to the control group were registered in this span. Thus, in the test group, the proportion of those who had *NFKB1* promoter region methylated by 0–10% was 100%, while in the control group this figure equaled 87%. At the same time, in the test group, there were 50% and 49% of those whose *NFKB1* promoter region was hypomethylated (0% methylation) and lightly methylated (methylation up to 5%), respectively, and in the control group these figures were 63% and 23%, respectively. As for the *MDM2* gene, the bulk of differences between test and control groups was also in the 0–5% span, with hypomethylation registered in 29% of the test group cases and light methylation (0–5%) in 62%, while in the control group promoter region of *MDM2* was hypomethylated in 55% of participants and lightly methylated in 41%. For *BAX*, the trend was similar: in 98% of test group participants, the level of methylation was below 10%, and 2% exhibited hypermethylation (50 through 75%) of this gene promoter region. It is worth noting that in the control group, we registered all the designated spans of level of methylation of BAX's promoter region.

Given the relative uniformity of distribution by the levels of methylation, we divided the sample into two groups: group 1, methylation up to 10%; group 2, methylation over 10% (Table 3).

Table 2. Characteristics of the used oligonucleotides

Gene	Primer sequences (5'-3')	Number of CpG sites	Amplicon length	Primer length	Ta
BAX	F: GAGGGGTAGAAATTTTCGGAT R: ATAATACGAACGACAAACCCG	10	181	21 21	59
MDM2	F: TTTGTCGGGTTATTAGTGTGAAC R: CCTTTTACTACAATTTCGAAACGTA	6	130	23 25	60
TP53	F: GTAGTTTGAACGTTTTTATTTTGGC R: CCTACTACGCCCTCTACAAACG	11	135	25 22	61
NFkB1	F: GTAGGAAGAGGAGGTTTCGTTATC R: ACCGATAACTACGTACAAACCGA	14	122	24 23	60

Note: F — forward primer; R — reverse primer; Ta — annealing temperature.

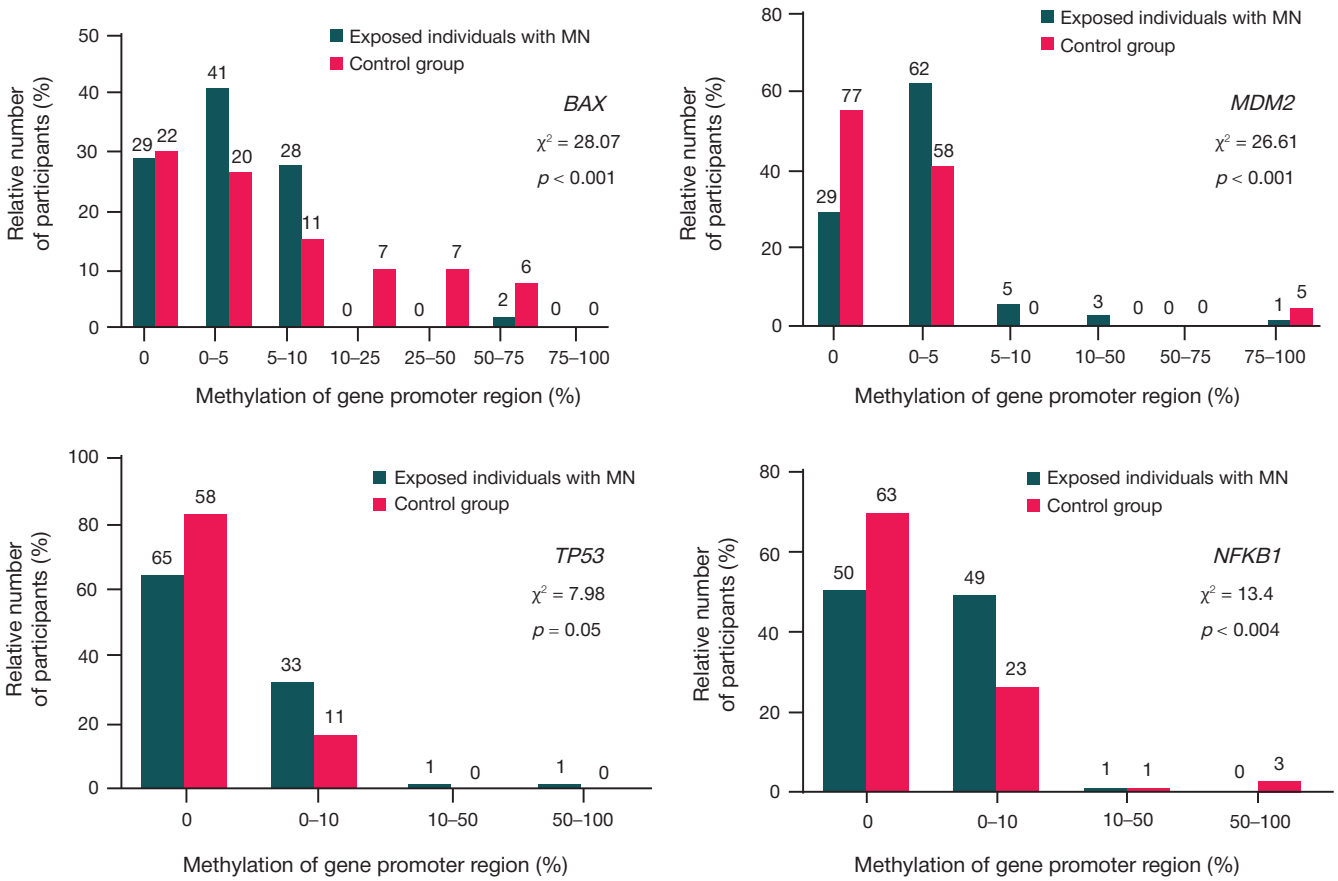

Fig. Distribution of the participants by level of methylation of CpG-dinucleotides of the studied gene promoter regions. Chi-squared test value incorporates Yates correction

We revealed significant differences only for *BAX*, the pro-apoptotic gene. Compared to the control group, the share of test group participants who had its promoter regions methylated for up to 10% was significantly higher ($p < 0.00001$).

Methylation is a dynamic process that may depend on a number of factors, including age and radiation dose. With this in mind, we correlated the levels of methylation with RBM dose and age at the time of examination. This analysis revealed no dependence of the methylation pattern on RBM dose and age in the test group, and in the control group, we registered a weak negative correlation between *BAX* and TP53's promoter regions methylation and age of the participants ($R = –0.35$; $p = 0.002$ and $R = –0.28$; $p = 0.02$, respectively) (Table 4).

DISCUSSION

The subjects of this study were the cell cycle (*MDM2*, *TP53*) and apoptosis (*BAX*, *NFkB1*) genes. By distribution of the levels of methylation of *BAX*, *MDM2* and *NFkB1* promoter regions, test group (exposed individuals, subsequent MN) differed significantly from the control group (exposed individuals, no subsequent MN). However, having divided the sample into two groups by the degree of methylation (up to 10% and above 10%), we discovered statistically significant differences only for *BAX*. In the test group, 98% of the participants had the levels of methylation between 0 and 10%, while in the control group, this figure did not exceed 73%.

BAX is a member of BCL-2 family; it induces apoptosis and is considered a potential tumor suppressor [23]. Normally, in response to genotoxic damage, the p53 protein alters the level of expression of genes involved in mitochondrial-mediated apoptosis, and activates *BAX*, inter alia [24]. At the same time, tumor cells suppress pro-apoptotic genes, seeking to survive and metastasize. It is important to note that decreased concentration of the BAX protein is associated with mutations in the *Tp53* gene [25]. According to our studies, exposed individuals with latent MN have *BAX* promoter in blood cells hypomethylated, which may affect the transcriptional activity of this gene. It is interesting

Table 3. Cases of methylation of CpG islands of the promoter regions of *BAX*, *MDM2*, *TP53*, *NFkB1* in the study sample

Gene	Level of methylation	Control group N (%)	Exposed individuals with latent MN N (%)	*p*-value
BAX	0–10%	53 (72.6)	98 (98)	$p < 0.00001$
	Over 10 %	20 (27.4)	2 (2)	
TP53	0–10%	69 (100)	98 (98)	$p = 0.51$
	Over 10 %	0 (0)	2 (2)	
MDM2	0–10%	135 (96.4)	96 (96)	$p = 0.99$
	Over 10 %	5 (3.6)	4 (4)	
NFkB1	0–10%	87 (96.6)	100 (100)	$p = 0.10$
	Over 10 %	3 (3.6)	0 (0)	

Note: *p* — is the level of statistical significance of differences between groups, as given by Fisher's exact test.

Table 4. Spearman's rank correlation coefficients (R), dependence of the degree of methylation of the studied gene promoter regions on RBM dose and patient's age at the time of the study. The p-value for Spearman's correlation coefficients is given in parentheses

Gene	Control group		Exposed individuals with latent MN	
	RBM dose	Age at the time of examination	RBM dose	Age at the time of examination
MDM2	–0.03 (0.69)	0.09 (0.31)	–0.06 (0.53)	–0.05 (0.64)
BAX	–0.55 (0.64)	–0.35 (0.002)	0.08 (0.44)	0.08 (0.45)
TP53	–0.08 (0.53)	–0.28 (0.02)	–0.01 (0.99)	0.07 (0.48)
NFkB1	0.14 (0.19)	0.10 (0.34)	0.04 (0.70)	–0.02 (0.86)

to note that findings of a previous study that involved residents of the Techa riverside villages and investigated expression of mRNA of apoptotic genes: in those whose RBM dose exceeded 522 mGr, transcriptional activity of *BAX* was increased significantly [21]. Another study looked into death of peripheral blood lymphocytes in the same cohort, and found that in the exposed with obligate precancers, the level of respective apoptosis was higher than in those who were also exposed but had no precancer [26].

There is a sufficient number of published works investigating profiles of methylation of DNA in cancer cells, with colon, breast and lung cancers being the most common neoplasms considered [27]. At the same time, there are significantly fewer retrospective studies that look into methylation of DNA in normal tissues (for example, blood) before the onset of the disease, studies that seek cancer risk predictors; moreover, most of them consider genes associated with changes in the chronological age (epigenetic clock) [13, 15, 17]. There are, however, isolated studies of proto-oncogenes and tumor suppressor genes. One of them analyzed patterns of methylation of 17 genes potentially indicating predisposition to breast cancer, including cell cycle regulation genes, and found that, compared to the control group (no breast cancer), patients suffering the disease had the intragenic repeating element of the ATM gene hypermethilated [28].

Thus, the results of this study demonstrate that epigenetic modifications (degree of methylation) in the peripheral blood DNA can potentially be used as markers of radiation-induced carcinogenesis. In addition, identification of epigenetic changes in tissues and cells not involved in the pathological process allows clarifying the causes of pathological conditions. However, definitive determination of epigenetic markers of carcinogenic effects of radiation requires additional studies involving expanded samples and factoring in the analysis of the level of methylation registered in tumor tissues.

CONCLUSIONS

By distribution of the levels of methylation of *BAX*, *MDM2* and *NFkB1* promoter regions, test group (individuals exposed to chronic low dose rate radiation, with RBM doses from 10.1 to 3,507 mGy, latent MN) differed significantly from the control group. The share of the test group participants who had up to 10% of the *BAX* gene promoter regions methylated was significantly higher, and amounted to 98%, while in the control group this figure did not exceed 73%. There was revealed no dependence of the level of methylation of the studied gene promoter regions on RBM dose.

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FREQUENCY OF INVERSIONS IN THE T-LYMPHOCYTE CHROMOSOMES OF EXPOSED RESIDENTS OF THE SOUTHERN URALS

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It is well-known that ionizing radiation is among factors increasing the rate of chromosomal rearrangements. The inversion rate was poorly understood due to difficulty of inversion identification by the conventional differential staining method. A comprehensive study of chromatin and its complex rearrangements has become possible with the use of the high-tech molecular genetic method, fluorescence *in situ* hybridization (FISH). The study was aimed to assess frequency of inversions involving the chromosome telomeric regions in 36 residents of the South Urals, almost all of them were affected by combined chronic exposure. The calculated individualized cumulative external and internal doses were 0.0001–4.7 Gy. Inversions were identified by fluorescence staining of the chromosome telomeric region. It was found that chromatid inversions were more abundant than chromosomal variants (9 : 0.3 per 100 cells ($p < 0.001$). No relationship between the studied parameters and the absorbed dose, sex and age at the time of the examination was revealed.

Keywords: chromosomal aberrations, inversions, telomeric regions of chromosomes, ionizing radiation, FISH, Techa River

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Author contribution: Vozilova AV — study concept, research priority setting, literature review, manuscript writing; Krivoshchapova YaV — developing criteria for analysis, staining and slide assessment, statistics, manuscript writing.

Compliance with the ethical standards: the study was approved by the Ethics Committee of the Urals Research Center for Radiation Medicine (protocol № 7 dated 20 October 2023); individuals, who were through cytogenetic testing, submitted the informed consent to blood sampling and further assessment.

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ЧАСТОТА ИНВЕРСИЙ В ХРОМОСОМАХ Т-ЛИМФОЦИТОВ У ОБЛУЧЕННЫХ ЖИТЕЛЕЙ ЮЖНОГО УРАЛА

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Известно, что ионизирующее излучение — это один из факторов, повышающих частоту хромосомных перестроек. Распространенность инверсий была мало изучена из-за сложности их выявления общепринятым методом дифференциальной окраски. Комплексное изучение хроматина, его сложных перестроек стало возможно с применением высокотехнологичного молекулярно-генетического метода — флуоресцентной *in situ* гибридизации (FISH). Целью исследования было изучить частоту инверсий с вовлечением теломерных участков хромосом у 36 жителей Южного Урала, почти все из которых подверглись сочетанному хроническому облучению. Рассчитанные индивидуализированные суммарные дозы от внешнего и внутреннего облучения — от 0,0001 до 4,7 Гр. Инверсии выявляли методом флуоресцентной окраски теломерного участка хромосом. В результате обнаружили, что распространены преимущественно хроматидные инверсии по сравнению с хромосомными вариантами (9 : 0,3 на 100 клеток ($p < 0,001$). Не выявлено зависимости исследованных показателей от дозы облучения, пола и возраста на момент обследования.

Ключевые слова: хромосомные аберрации, инверсии, теломерные районы хромосом, ионизирующее излучение, FISH, река Теча

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Соблюдение этических стандартов: исследование одобрено этическим комитетом УНПЦ РМ (протокол № 7 от 20 октября 2023 г.), у лиц, участвующих в цитогенетических исследованиях, было получено информированное согласие на забор образцов крови и дальнейшие исследования.

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For more than 60 years individuals, chronically exposed due to the Mayak PA liquid radioactive waste releases into the Techa River (Southern Urals), have been undergoing medical examinations in the Urals Research Center for Radiation Medicine of the Federal Medical Biological Agency of Russia (URCRM). The long-term follow-up of the cohorts of exposed people leads the researchers to the understanding of the complex interaction of radiation and non-radiation factors and its further effects on human health. Studying the chronic exposure effects on the body remains an essential task for researchers and medical professionals, since it helps to reveal the mechanisms underlying the

effects of radiation and prevent adverse impact of radiation exposure [1].

Great amount of research is focused on exploring the mechanisms underlying the emergence of chromosomal mutations and identifying their role in evolution of species, implementation of the ontogenesis, effects on the organs and tissues of a human body [2, 3]. The exposure to ionizing radiation can trigger development of various biological effects, also including chromosomal aberrations [4, 5], translocations (stable aberrations), as well as ring and dicentric chromosomes (unstable aberrations) being the most thoroughly studied ones. Follow-up of the cohort of individuals affected by combined chronic exposure (hereinafter referred to as exposure) in the Southern Urals demonstrates high frequency of translocations and unstable chromosomal aberrations relative to background indicators even 70 years after the beginning of exposure [6]. Today, there are sporadic reports showing the direct correlation between the increased frequency of chromosomal rearrangements and diseases in humans. Some investigations consider cancer as an effect. The studies have shown that up to 70% tumor cells contain chromosomal rearrangements of various types [7].

In recent years, the researchers' attention was focused on exploring the chromatin packaging and behavior in the nucleus, since the range of methods suitable for such studies expanded. The scientists construct models and predict the effects of various factors and genetic mutations on the chromatin architecture based on the data on the frequencies of various types of chromosomal rearrangements and differentiated chromatin arrangement in the cell nucleus in the 3D format [8].

Chromatin, consisting of heterochromatin and euchromatin regions, has a complex structure and compaction. It is well-known that chromosomal rearrangements result in redistribution of these structures across the chromosome arms or different chromosomes in the nucleus or in elimination of certain regions, which inevitably affects expression of oncogenes, suppressor genes, etc., as well as cell functioning.

Among stable chromosomal aberrations, inversions are the most poorly understood, because these are difficult to identify. Inversions are chromosomal rearrangements in which the chromosome structure alteration is caused by the 180-degree turn of one of its regions. Inversions are divided into two classes: pericentric and paracentric. Pericentric inversion includes the centromere and changes the chromosome structure, which makes it easy to verify during karyotyping. Paracentric inversion is less easy to detect, since it does not change the ratio of the chromosome arms. The DNA breakage/fusion mechanism underlies the emergence of inversion [9].

Inversion plays an important biological role. According to the published data, inversions in chromosomes are most often found in the cells that are undergoing malignant transformation, in individuals with congenital syndromes associated with developmental delay, autism and epilepsy [10, 11]. It is well-known that inversion affects the occurrence of crossing over between sister chromatids and segregation of chromosomes into daughter cells, which can result in aneuploidy or cell death [12].

There are various methods to detect chromosomal inversion. Among cytogenetic approaches, G-banding (GTG-banding) is the most widely used and affordable one. However, the complex and time-consuming nature of the analysis made it impossible to widely use the approach to assess the abundance of various types of inversions in human cells. Fluorescence in situ hybridization, to be more specific high-resolution multicolour banding FISH (mBAND), is a modern high-tech method to determine chromosomal inversion [13–15]. This method is reliable, but rather expensive to study the population frequencies of inversions in human cells. We have tried to use FISH with locus-specific telomeric probes for this purpose [16]. When assessing the length of the metaphase chromosome telomeric regions, we sometimes detected fluorescence signals of telomeres within chromosome arms, which was indicative of the chromatin inversion involving the chromosome terminal regions. The pilot-stage findings of the study of the frequency of inversions involving telomeric regions were presented in our previous paper [16], however, to be confident in the obtained results it was necessary to expand the sample and then assess the relationship between the indicators and the radiation and non-radiation factors.

The objective of the study was to assess the frequency of inversions involving the metaphase chromosome telomeric regions in T-lymphocytes of individuals affected by combined chronic exposure on the Techa River. To accomplish this objective a task was set to assess the relationship between the frequency of inversions and the cumulative external and internal dose, as well as the age at the time of examination and sex.

METHODS

Characteristics of examined individuals

The study involved residents of the Southern Urals born before 1960, the majority of them had cumulative absorbed doses to RBM (red bone marrow) of 0.0001–4.7 Gy (calculated according to the TRDS-2016). These individuals were either members of the Techa River Cohort (TRC) or Techa River In Utero Exposed Cohort (TRCIU). Information about the studied sample and health status of exposed individuals was provided by the "Database "Man" Department. Individualized cumulative external and internal doses (hereinafter referred to as doses) to RBM were calculated using the TRDS-2016 in the Biophysics laboratory, the data on the history of cancer in the examined individuals were provided by the Epidemiology Laboratory of the Urals Research Center for Radiation Medicine (URCRM) [1].

The fact of combined exposure (internal β- and external γ-exposure in a wide dose range) was the specific feature of chronic exposure of the residents of the Techa Riverside villages. A total of 29 females and 7 males were examined during the study. Inclusion criteria: age 61–81 years. Ten donors had high absorbed doses to RBM (1–4.7 Gy), 12 individuals had the absorbed doses to RBM within the range of 0.3–0.9 Gy. The comparison group included two non-exposed individuals and 12 exposed individuals with the absorbed doses to RBM of 0.0001–0.01 Gy.

Exclusion criteria: individuals born in 1961 and later; history of autoimmune diseases, cancer, exacerbation of chronic inflammatory diseases. People taking cytostatics, antibiotics were not included. The characteristics of studied groups are provided in Table 1.

Obtaining the peripheral blood T-lymphocyte metaphase chromosome preparations

The cytogenetic study involved metaphases of the peripheral blood T-lymphocytes stimulated with phytohemagglutinin (PHA). The chromosome preparations were obtained in accordance with the protocol including four consecutive stages: cell culturing to the metaphase stage, hypotonic treatment of cells, metaphase plate fixation and making

Table 1. Characteristics of studied groups

Dose group, Gy	Number of donors (total) Age, years		Females		Males	
	Number, *n*	Age, years	Number, *n*	Age, years	Number, *n*	Age, years
0–0.01	14	62–72	10	62–72	4	62–70
0.3–0.9	12	69–81	11	69–81	1	72
1–4.7	10	70–76	8	70–76	2	71–72
Total	36	62–81	29	62–81	7	62–72

the chromosome preparations [17]. When drops of the cell suspension were pipetted onto the slides, slides were dried at 42 °C on the slide dryer, then stored in the freezer at –20 °C prior to fluorescent staining.

Telomeric region staining by fluorescence in situ hybridization (FISH) with locus-specific probes

Chromosomal inversions involving telomeric regions were assessed using the Telomere FISH Kit/Cy3 telomeric probes (Dako; Denmark). The Cy3-conjugated peptide nucleic acid used to produce the probe is synthetic DNA analog capable of binding to DNA of chromosomes in accordance with the base pairing rules. In peptide nucleic acid, the sugar-phosphate backbone is replaced with the neutral peptide-polyamide backbone, however, the distance between base pairs remains the same as in DNA. It is important to note that the probe from this set does not recognize subtelomeric chromatin sequences and therefore enables staining of the chromosome telomeric regions only [18]. Chromosomes were stained in accordance with the protocol of the probe manufacturer. The fluorescence-stained preparations were analyzed using the Axio Imager Z2 microscope (Zeiss; Germany) with the DAPI and SpO (Spectrum Orange) filter and the Isis software package. Metaphases containing 46 chromosomes without overlapping or artifacts were included in the analysis. All the chromosomes were analyzed in each cell to find inversions. A total of 100 cells per donor were counted, a total of 3,600 cells were analyzed during the study. We estimated total number of inversions by types and the sum of all inversions per 100 cells, as well as the group-average values. Since the criteria of dividing inversions into chromatid and chromosomal were discussed in detail in previous report [16], here we provide a brief reminder of the mechanisms underlying the emergence of inversions and various inversion types (Fig. 1, 2).

Statistical analysis

The results were analyzed using the STATISTICA 10 software package (StatSoft; USA). Statistical processing of the results was performed using the nonparametric Mann–Whitney U test.

RESULTS

Frequency of chromosomal inversions in the range of 0–2 was reported in 9 individuals, while chromatid inversions in the range of 3–26 were reported in all examined individuals. In non-exposed individuals (2 people), the chromatid inversion frequency was 6 and 19% and the chromosomal inversion frequency was 0 and 1%, respectively. The ratio of average frequencies of chromatid and chromosomal inversions was 9 : 0.3 per 100 cells ($p < 0.001$) (Table 2).

As it is shown in Table 2, frequency of inversions in the studied groups with increasing absorbed dose to RBM demonstrates no significant differences. Low values were observed in individuals with the highest doses of 1–4.7 Gy. Maximum values were typical of chromatid inversions (frequency was 9.2, 9.5 and 8.7, respectively). The chromosomal inversion frequency was between 0.4 in the first two dose subgroups and 0.2 in the subgroup of individuals exposed at high doses.

The dependence of the inversion frequency on the age at the time of examination is provided in Fig. 3.

Therefore, no age-dependence of the inversion frequency was found in the studied age range (60–80 years).

No dependence of the inversion frequency on the absorbed dose to RBM was revealed either.

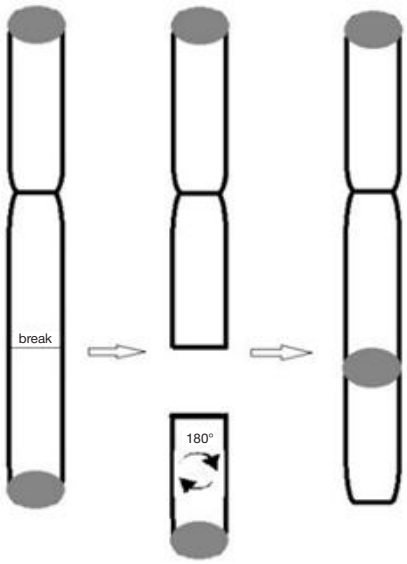

Fig. 1. Potential mechanism underlying the emergence of inversion involving the chromosome telomeric region

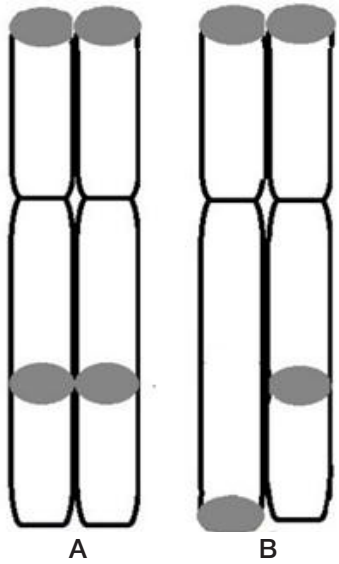

Fig. 2. Inversion types: chromosomal (**A**) and chromatid (**B**) (telomeric region is highlighted in *gray*)

Table 2. Frequency of inversions (M ± SD) (median, 25 and 75%) involving telomeric regions in T cells of exposed residents of the Southern Urals (per 100 cells)

Dose groups, Gy (*n*)	Chromatid inversions M ± SD, Median, (25–75%)	Chromosomal inversions M ± SD, Median, (25–75%)	Total inversions M ± SD, Median, (25–75%)
Comparison group (13)	9.2 ± 4.7 9 (6–11)	0.4 ± 0.7 0 (0–1)	9.6 ± 5.1 9.5 (6–12.5)
0.3–0.9 (11)	9.5 ± 6.0 8 (6.5–9)	0.4 ± 0.2 0 (0–0)	9.6 ± 6.0 8 (7.5–9)
1.00–4.7 (11)	8.7 ± 3.7 7 (6–10.5)	0.2 ± 0.4 0 (0–0)	8.3 ± 4.8 7 (6–10.5)
Entire group (36)	9.1 ± 4.8 8.5 (6–11)	0.3 ± 0.5 0 (0–0.25)	9.4 ± 5.0 8.5 (6–11)

There were few men in the studied sample. That is why, to assess the sex effect on the studied parameter a group of women was formed. Women were selected in accordance with the case-control principle for each examined man taking into account the absorbed dose to RBM and age (Table 3).

Thus, no dependence of the inversion frequency on the sex of the examined individuals was revealed.

DISCUSSION

The study reported in the paper is a continuation of a pilot project started more than two years ago in the Laboratory of Radiation Genetics, Urals Research Center for Radiation Medicine as part of the Russian Foundation for Basic Research grant. During this project we assessed the metaphase chromosome inversion frequency in the cultured peripheral blood T cells of exposed residents of the Southern Urals [16]. For this purpose a method of fluorescent staining of the chromosome telomeric regions was proposed and tested. In the given paper the size of the examined individuals sample was increased and the impact of radiation and non-radiation factors on chromosomal rearrangements (inversions involving telomeric regions of chromosomes) was analyzed. It has been found that thanks to the increase in the sample size the earlier reported frequencies of inversions have been confirmed: chromatid inversions were the most abundant and their ratio to chromosomal inversions was 9 : 0.3.

It is obvious that chromatid inversion is formed in one of the sister chromatids after the cell is through the synthesis phase of cell division, while chromosomal inversion results from the inversion emerged before the synthesis phase, which eventually causes duplication of the inverted chromatid during this phase. In this case the ends of the sister chromatid arms are left without telomeric regions, which is a marker of the cell death. This thesis is proven by lower frequencies of chromosomal inversions. If cells could survive such rearrangements, we would see high frequencies of chromosomal inversions or other chromosome rearrangements (for example, translocations or di- or multiple-centric chromosomes, ring chromosomes). However, this was not observed when assessing preparations.

The analysis of the published papers gives reasons to believe that there are some mechanisms that eliminate damaged chromosomes or cells, which makes it possible to preserve integrity of chromosomes in the cell and the genome in all cells of the body.

Our findings show that a large number of inversions contain telomeric repeats. The analysis of the published papers has allowed us to find reports noting that telomeric sequences are found in the chromosomal chromatin of many organisms, including human beings, and are referred to as interstitial telomeric sequences (ITSs) [19, 20]. Such regions are considered to result from genomic rearrangements during the course of karyotype evolution, which emphasizes the importance of studying these regions. There are assumptions explaining the mechanisms underlying the telomeric region insertion during repair. It is believed that short telomeric repeats can be inserted by the double-strand break repair systems involving telomerase [21] or result from replication induced by the double-strand

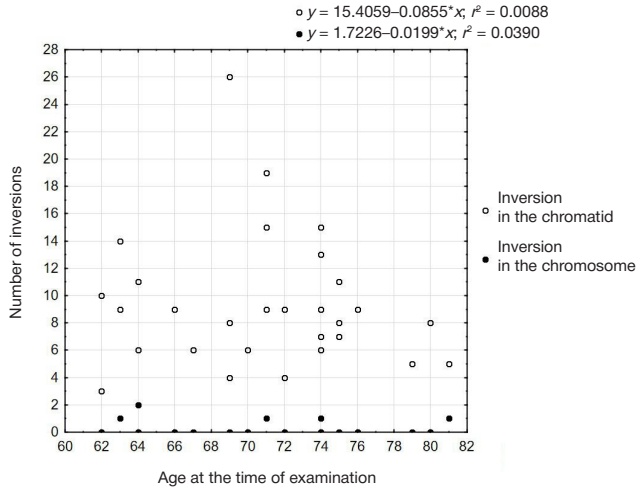

Fig. 3. Graph of inversion frequency versus age

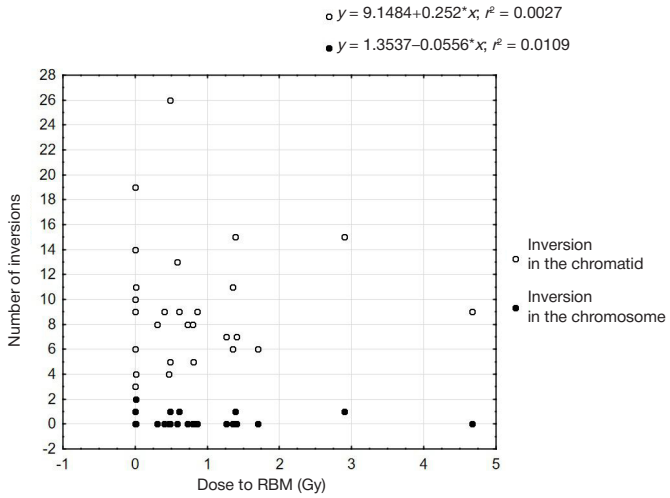

Fig. 4. Inversion frequency versus absorbed doses in the examined individuals

Table 3. Inversion frequency versus sex (M ± SD) per 100 cells

Sex (*n*)	Age, years	Dose, Gy	Chromatid inversions	Chromosomal inversions	Total inversions
M (7)	61–72	0.003–1.35	8.3 ± 4.3	0.1 ± 0.4	8.4 ± 4.6
F (7)	62–75	0.0001–1.35	7.7 ± 3.4	0.3 ± 0.5	8 ± 3.8

breaks or targeted insertion of telomeric sequences [22] based on the mechanism of alternative lenghthening of telomeres involving some homologous recombination components [23]. The loops formed by telomeric sequences are an important component of three-dimensional chromatin organization in the nucleus, which, in turn, is an important aspect of functional regulation of all processes in the genome [24]. Thus, ITSs can mediate telomeric regulation of the genome regions located far from the telomeres.

Considering the fact that the mechanism underlying inversion is the same as that underlying translocation (DNA breakage/fusion), it can be assumed that deletion of genes encoding proteins ensuring the chromosome end stabilization (such as TRF2) occurs due to the effects of regulatory mechanisms, which results in the chromosomal rearrangement. Consequently, the chromosome can either be eliminated, form a ring or "escape" via inversion. The single-stranded telomeric sequence, once inside the chromosome, is probably completed by telomerase, which is triggered by the interaction between the RNA matrix and the single-stranded primer. Telomerase adds nucleotides to the primer following the order dictated by the matrix structure [25].

Previously, we have reported the frequencies of chromosomal aberrations obtained for the group of individuals exposed to high-dose radiation in the Southern Urals [16]. Thus, during the analysis of chromosome preparations, chromatid inversions were the most abundant (9%), simple translocations accounted for 5%, complex translocations for 0.6%, and chromosomal inversions were the least abundant (0.3%). Given that each chromosome occupies a strictly defined space in the nucleus and normally does not overlap with chromatin of other chromosomes, the fact that the most frequent alterations are found within a single chromatid (chromosome) becomes quite explicable [8]. Thus, it is well-known that up to 55,000 single-stranded DNA breaks, which are mostly repaired, occur in the human cell. However, we have confirmed that when there are some chromatin loop structural disruptions, a chromatid inversion occurs during repair. It is clear that when it comes to exchange of regions between different chromosomes, simple translocations are more probable than complex rearrangements involving simultaneous breaks in different chromosomes and their close proximity to the repair systems. Our findings show that such rearrangements are 10 times less abundant than simple translocations. Rare findings of chromosomal inversions indirectly confirm our assumption that such aberrations are lethal to the cell or such chromosomes are eliminated during cell division. This thesis requires further confirmation.

Thus, we believe that further investigation of the cell nucleus chromatin structure, specifically chromosomal inversions, is important for understanding how genes interact with one another and what biological mechanisms underly such interaction at the chromosome level.

CONCLUSIONS

The frequency of inversions involving the T-lymphocyte chromosome telomeric regions in the sample of residents of the Southern Urals affected by combined chronic exposure with the absorbed doses to RBM between 0.0001 and 4.7 Gy was 1–26 per 100 cells.The ratio of chromatid inversion frequency to chromosomal inversion frequency is 9 : 0.3 per 100 cells. No relationship between the chromatid and chromosomal inversion frequency and the cumulative absorbed dose to RBM has been revealed.No relationship between the chromatid and chromosomal inversion frequency and age within the range of 60–80 years and sex has been revealed.

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EFFECT OF CYSTAMINE ON GASTRIC PROPULSIVE FUNCTION AND GAS EXCHANGE IN THE RAT MODEL OF RADIATION-INDUCED MYELOABLATION

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Radiation exposure of recipients before hematopoietic stem cell transplantation can cause gastrointestinal (GI) stasis. It is associated with complications of myeloablative radiation therapy: delayed vomiting, excess bacterial growth, endotoxicosis, systemic inflammation, and sepsis. The study was aimed to assess the possibility of GI stasis prevention by intragastric administration of cystamine dihydrochloride when using radiation-induced myeloablation. The severity of GI stasis, levels of enterocyte markers in the small intestinal tissues and the indicator of intestinal endotoxicosis, urinary indican excretion, were assessed in rats 72 h after the single total-body X-ray exposure to the dose of 9.64 Gy (1.1 $LD_{99/30}$); the animals' whole body oxygen consumption was recorded daily. Irradiation caused GI stasis with predominant gastric stasis, the 1.5–4.8-fold decrease in the cholinesterase and alkaline phosphatase activity in the small intestinal tissues, doubled the urinary indican excretion, the whole body oxygen consumption reduction by 17–32%. Cystamine administration generally prevented gastric stasis, but had no significant effect on the characteristics of radiation-induced enterocytopenia and did not prevent accumulation of chyme in the *caecum*, hyperindicanuria, radiation-induced spleen hypotrophy, and decrease in gas exchange rate. Cystamine is promising for testing in large animals as a selective agent for emergency prevention of gastric stasis during myeloablative radiation therapy.

Keywords: rats, radiation myeloablation, cystamine, gastrointestinal stasis, gastric stasis, indican, enterocytopenia, gas exchange

Author contribution: Vakunenkova OA — experimental procedure; Ivnitsky JuJu — rationale, developing the experimental model, data interpretation and discussion; Danilova OA — tissue biochemistry studies; Schäfer TV — experimental procedure, data processing and visualization, developing the experimental model; Rejniuk VL — methodological guidance of gas exchange assessment. All authors contributed to discussion, manuscript writing and editing.

Compliance with the ethical standards: the study was carried out in accordance with the principles of bioethics, approved by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

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ВЛИЯНИЕ ЦИСТАМИНА НА ПРОПУЛЬСИВНУЮ ФУНКЦИЮ ЖЕЛУДКА И ГАЗООБМЕН У КРЫС ПРИ ЛУЧЕВОЙ МИЕЛОАБЛЯЦИИ

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Облучение реципиентов перед пересадкой стволовых кроветворных клеток способно вызвать желудочно-кишечный стаз (ЖКС). С ним связаны осложнения лучевой миелоабляционной терапии: поздняя рвота, избыточный бактериальный рост, эндотоксикоз, системное воспаление и сепсис. Целью работы было оценить возможность предупреждения ЖКС при лучевой миелоабляции профилактическим введением в желудок цистамина дигидрохлорида. У крыс определяли выраженность ЖКС, содержание маркеров энтероцитов в тканях тонкой кишки и показатель кишечного эндотоксикоза — экскрецию индикана с мочой — через 72 ч после общего однократного рентгеновского облучения в дозе 9,64 Гр (1,1 $ЛД_{99/30}$); ежедневно регистрировали потребление животными кислорода. Облучение вызывало ЖКС с преобладанием гастростаза, снижало активность холинэстеразы и щелочной фосфатазы в тканях тонкой кишки в 1,5–4,8 раза, вдвое повышало экскрецию индикана с мочой, на 17–32% снижало потребление кислорода организмом. Введение цистамина в основном предупреждало гастростаз, но не оказывало существенного влияния на показатели лучевой энтероцитопении, не предупреждало накопление химуса в слепой кишке, гипериндиканурию, лучевую гипотрофию селезенки и снижение интенсивности газообмена. Цистамин перспективен для апробации на крупных животных в качестве селективного средства экстренной профилактики гастростаза при лучевой миелоабляционной терапии.

Ключевые слова: крысы, лучевая миелоабляция, цистамин, желудочно-кишечный стаз, гастростаз, индикан, энтероцитопения, газообмен

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The term "myeloablation" was proposed in 1952 to define irreversible pancytopenia following the single total body X-ray exposure to a supralethal dose [1]. Radiation-induced myeloablation has found application in clinical practice: it is used to prepare recipients for hematopoietic stem cell transplantation; such preparation is referred to as "conditioning" [2]. Irradiation involving 1–3 fractions with the total doses of 8–12 Gy and transplantation of hematopoietic stem cells after 2–5 days is used for radical treatment of hemoblastoses, some solid tumors, myelodysplastic and autoimmune disorders [3]. In

individuals with acute leukemia, radiation-induced myeloablation is used solo or in combination with chemotherapy. In the latter case, it is considered as a method to combat chemoresistance in cancer cell clones [4]. Radiation-induced myeloablation is the main method to treat T-cell acute lymphoblastic leukemia in children and adults [5, 6]. Irradiation with myeloablative doses can also occur outside the clinic: during the first stage of the nuclear power reactor accident, under exposure to penetrating radiation of a nuclear explosion, when staying in the zones of dangerous or extremely dangerous radioactive contamination of the terrain with the nuclear explosion products [7].

The most prevalent and severe complications of myeloablative therapy are represented by the disorders referred to as "oral mucositis" and "gastrointestinal toxicities" in foreign clinical trials [8, 9]. Impaired regeneration of the gastrointestinal tract mucosal epithelium is a common pathogenetic basis of these disorders. Among organs of the gastrointestinal tract, damage to the small intestinal epithelium is the most important. That is why selective radiation shielding of the small intestinal mucosa seems to be a promising approach to prevention of the myeloablative radiation therapy gastrointestinal complications.

One of those is gastrointestinal (GI) stasis, the reversible dose-dependent slowing of the gastrointestinal transit of chyme. There are few reports of such clinical cases, however, the possibility of modeling GI stasis by exposure of rats [10], guinea pigs [11], dogs [12] and monkeys [13] to the doses exceeding 1 Gy suggests that GI stasis comlplicates myeloablative radiation therapy more often, but under the "mask" of other diagnoses. GI stasis occurred in 26% of recipients after the end of primary acute radiation-induced response and manifested itself in the form of nausea, vomiting, bloating and distension of the stomach; it was confirmed by scintigraphy [14].

The GI stasis clinical significance is determined by its influence on the radiation exposure outcome: it hampers the patients' nutrition, makes it pointless to prescribe oral drugs, contributes to damage to the gut-blood barrier with the influx of lipopolysaccharides of Gram-negative bacteria into the blood and the development of sepsis [15]. Its accompanying overgrowth of gastrointestinal microbiota results in realization of the quorum sensing effect, intensification of generation of toxic substances by bacteria, endotoxemia and endotoxicosis [16]. Some of these substances show pulmonary toxicity, and the stomach congested with chyme can limit diaphragmatic excursion. In some recipients, X-ray gastric shadow spreads to large parts of both abdominal and thoracic cavities after the course of myeloablative therapy [17]. That is why abnormal external respiration and gas exchange are potential effects of gastric stasis.

Perhaps, GI stasis is a defensive response to acute radiation-induced mucositis, the main pathogenetic link of which is represented by cytopenia. In this regard, it can be assumed that the drugs preventing cytopenia, radioprotectors, can prevent GI stasis. Of greatest interest are indralin, one of modern standard radioprotective agents [18], and cystamine dihydrochloride that has been earlier used as a radioprotective agent. The latter remained the only sulfur-containing radioprotector registered in our country for a long time; in 1960–2012, it was part of first-aid and sanitation kits for the military unit of the medical service of the Russian Armed forces. There is an experience of using the substance in clinical practice [19]. Despite the fact that this drug is not listed in the State Register of Medicines as at 20 November 2023, it seems reasonable to test the drug as an agent for pathogenetic prevention of radiation-induced GI stasis. The study was aimed to test the hypothesis that cystamine dihydrochloride administered by intragastric route prevented GI stasis, endotoxicosis and gas exchange abnormalities in the rat models of radiation-induced myeloablation.

METHODS

The study involved male Wistar rats (161–190 g), purchased from the Rappolovo laboratory animal nursery. The diet included standard rat food and ad libitum access to water. Animals were randomized into experimental groups. To be deprived of food, rats were placed in the slatter floor cages, preventing coprophagy and consumption of the bedding components, with access to water only.

The time period of myeloablative conditioning was an order of magnitude shorter than the half-time of recovery after radiation exposure (25–45 days in humans). That is why, despite the fact that the myeloablative exposure dose is usually fractionated, the effective value does not differ significantly from the value of the sum of fractions. Therefore, radiation-induced myeloablation was modeled by the single X-ray irradiation in the multifunctional mobile X-ray apparatus (ELTECH-MED; Russia). Rats were placed in the polyethylene terephthalate pencil cases (eight rats per pencil case) positioned radially head to center in the circular polymethyl methacrylate rack. Irradiation parameters: focal range — 564 mm; anode voltage — 60 kV; anode current — 13 mA; filter: polymethyl methacrylate 8 mm + polyethylene terephthalate 0.4 mm; absorbed dose to the geometric center of the body — 9.64 Gy (1.1 $LD_{99/30}$). This dose was identified based on preliminary assessment of the dose dependence of the average life expectancy of exposed rats as a maximum dose, with which life expectancy of all animals was at least 3 days after exposure; in humans this corresponds to the average time period of myeloablative conditioning. The radiation dose rate was 0.27 Gy/min. The minimum to maximum body's exposure dose ratio was 0.9 in the caudo-cranial and 0.5 in the ventro-dorsal direction. Irradiation that lasted for 52 min was applied in three fractions of 12 min with two intervals of 8 min. In preliminary experiments, such exposure resulted in the development of pancytopenia syndrome after 3 days, reduction of relative weight of the spleen by 62 % and femoral bone marrow by 41 %, DNA density in these tissues by 2 and 1.9 times, respectively, as well as in the animals' death after 5.9 ± 1.5 days (M ± *m*, *n* = 16).

Laparotomy and organ harvesting were performed under the mask halothane anaesthesia. The GI stasis severity was assessed based on the relative weight of chyme in the stomach and *caecum* calculated as a difference between the weight of the organ filled with chyme and the weight of the empty organ (*gaster*, *caecum*) in grams relative to body weight in kilograms.

In the first phase of the study we assessed the dynamics of GI stasis following myeloablative conditioning. For that animals were divided into eight groups, among them four were represented by animals deprived of food 2, 24, 48 or 72 h after exposure, while the other (control) ones were represented by animals deprived of food within the same time frame, but non-exposed and provided unlimited access to water. After 72 h severity of GI stasis in animals was assessed.

In the second phase we assessed the cystamine dihydrochloride effects on the GI stasis severity, growth rate of gastrointestinal microbiota and the levels of enterocyte markers in the small intestinal tissues. We used rats having unlimited access to water, but deprived of food between 24 and 72 h after exposure. Animals were divided into three groups, among which the first group was represented by intact animals not receiving cystamine and other groups consisted of exposed animals. Animals of the third group received intragastric injection of 10

mL/kg of the cystamine dihydrochloride (synthesized in the State Scientific Research Test Institute of the Military Medicine of Defense Ministry of the Russian Federation) aqueous solution in a dose of 120 mg/kg 30 min before the beginning of irradiation. This dose, based on the body weight to body surface area ratio of humans, is bioequivalent to the drug dose of 1.2 g prescribed to be taken 30–40 min before irradiation, i.e. to the content of the case contained in individual first-aid kits AI-1, AI-1M and AI-2. Rats were placed in metabolic cages for urine collection 48 h after the exposure. Animals were subjected to laparotomy 72 h after the exposure to assess the GI stasis severity, proximal sections of the *duodenum*, *jejunum* and distal sections of the *ileum* were retrieved. To assess the radioprotector effect selectivity, relative weight of the spleen was determined along with the relative weight of the *gaster* and *caecum* chyme as a measure of myeloprotective effect.

In the third phase we assessed the dynamic changes in the gas exchange and external respiration indicators for 3 days after irradiation of unprotected animals or animals receiving cystamine.

The gastrointestinal microbiota growth rate was assessed based on the urinary indican excretion. The levels of indican in the urine collected within 24 h were determined by the quantitative colorimetric method [20]; indican excretion was expressed in micrograms per kilogram of body weight per hour.

Enterocytopenia was quantified based on the enterocyte membrane marker activity in the tissues of the *duodenum*, *jejunum* and *ileum*: cholinesterase (ChE) and alkaline phosphatase (ALP). The small intestinal segments with the length of 4 cm were weighed, homogenized in the 15-fold larger volume of the Tris-HCl buffer solution (50 mmol/L, pH 7.4), frozen at –20°C, thawed 15 h later at 4°C and centrifuged for 10 min at 2000 g. The supernatant protein content was determined using the Bradford assay. The ChE activity was determined by the Ellman's method in the ChemWell 2910 biochemical analyzer (Awareness Tech.; USA) using acetylthiocholine iodide as a substrate. The ALP activity was measured by the kinetic method using the reagent kit (Olvex Diagnosticum LLC; Russia) at 37°C in the ChemWell 2910 biochemical analyzer (Awareness Tech.; USA).

The whole body oxygen consumption was determined in the apparatus constructed by Miropolsky. Animals were habituated to the respirometry chamber for two days before the beginning of the study. The following equation was used to calculate the whole body oxygen consumption (Q_{O2}, mL/(kg · min)):

$$Q_{O2} = V \cdot F/(m \cdot \Delta t),$$

where V was the volume of manometric fluid in the burette, mL; F was a coefficient used to adjust the oxygen volume to standard conditions; m was the aminal's body weight, kg; Δt was the length of the rat's stay in the sealed chamber, min.

The duration of measurement was 3 min, its absolute error was 0.1 mL (≤ 2 % of V value), and the respirometry chamber volume was 0.9 L. Animals were not secured, they could move freely in the respirometry chamber and looked dazed. During this time the animals' respiratory rate (RR, min^{-1}) was determined, which was considered as a measure of external respiration. The whole body average oxygen consumption per respiratory cycle (mL/kg) calculated as a ratio of Q_{O2} to RR was used as a measure of the external respiration efficiency. The Q_{O2}, RR and Q_{O2}/RR values calculated after irradiation were expressed as a percentage of baseline level taken as 100%.

The results were presented as mean and error of the mean (M ± *m*). The effects of radioprotector on the studied quantitative characteristics were assessed using analysis of variance. When the differences obtained were significant, the intergroup comparison of mean values was performed using the Tukey's honest significance test. The correlations between characteristics were represented as the Spearman's rank correlation coefficients (r_s). The α-value of 0.05 was considered to be a critical significance level.

RESULTS

In rats deprived of food within 48 h before laparotomy, the stomach that was dilated and filled with chyme occupied most of the abdominal cavity 72 h after irradiation; it looked empty in intact animals. The volume of the caecum increased to the lesser extent after the exposure (Fig. 1). Food consumed after irradiation accumulated in the stomach throughout the time of observation, which resulted in the progressive increase in

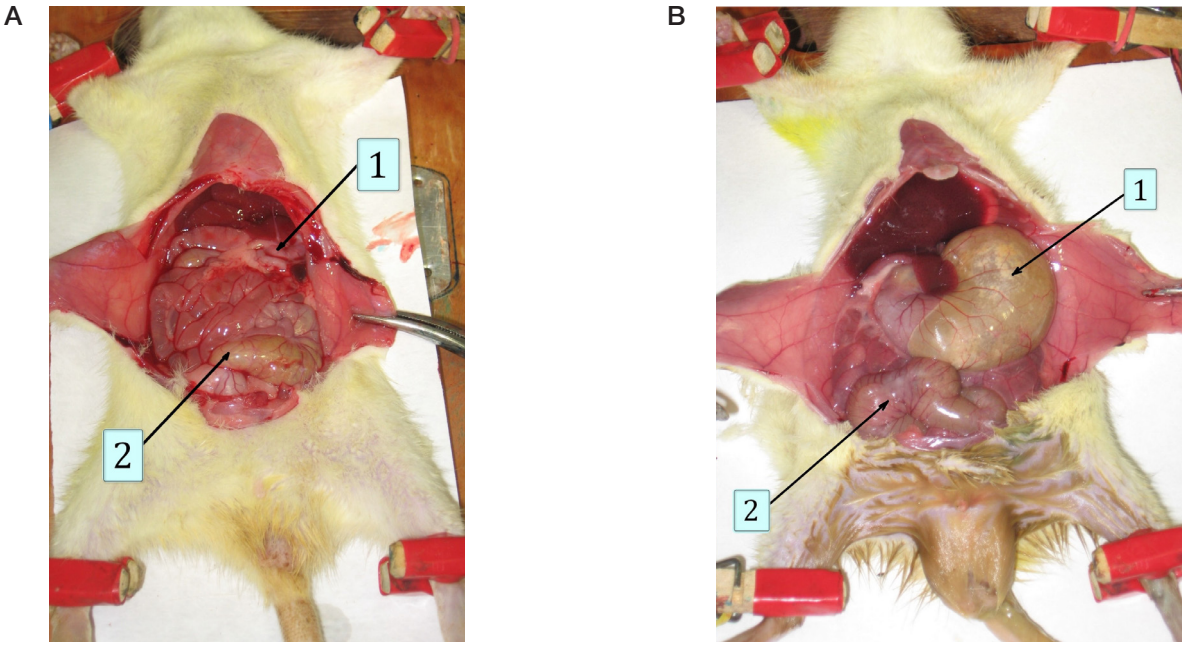

Fig. 1. Abdominal organs of rats deprived of food 48 h before laparotomy. **A**. Intact. **B**. 72 h after single total body X-ray exposure at a dose of 9.64 Gy. Arrows: 1 — stomach; 2 — *caecum*

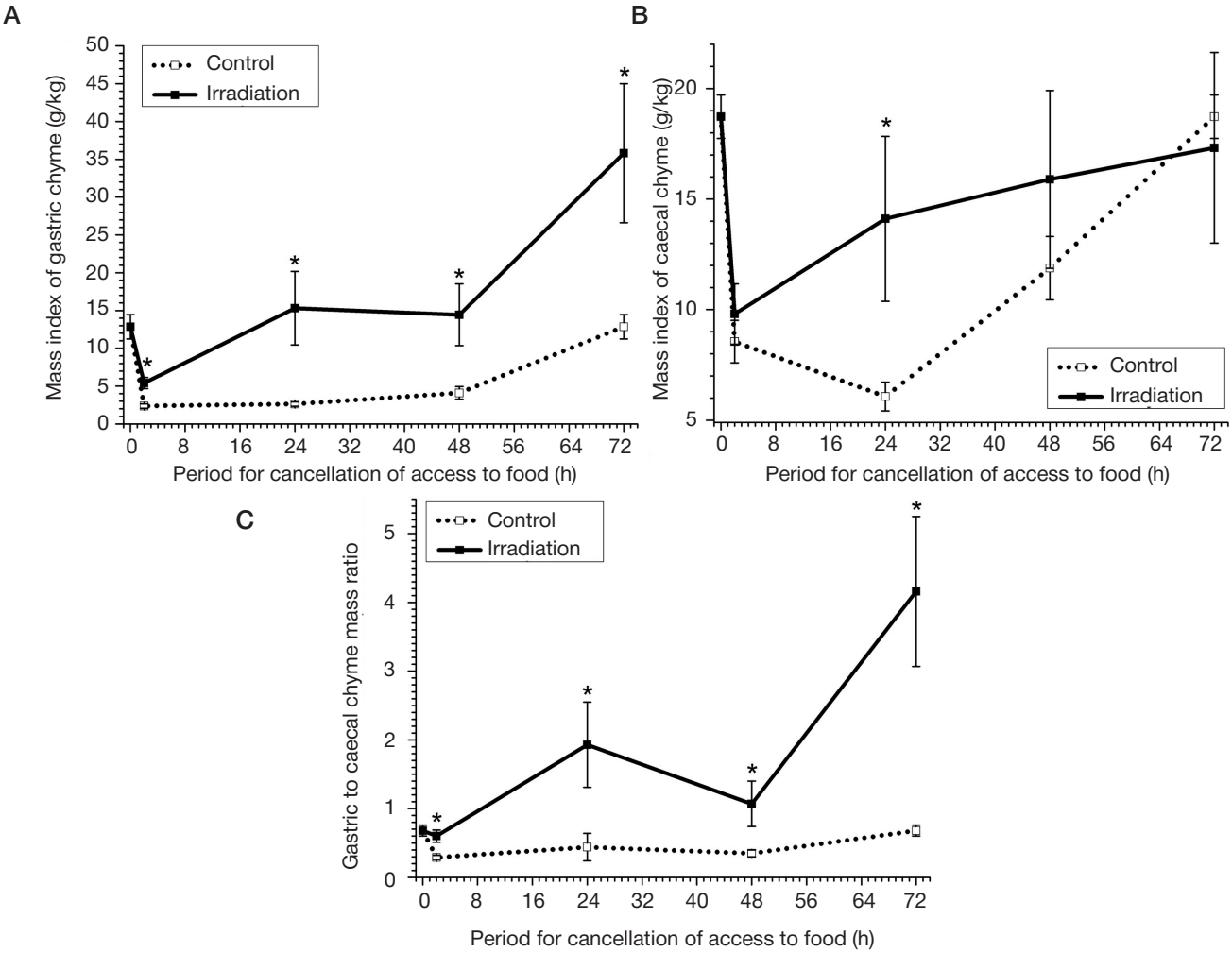

Fig. 2. Mass indexes of gastric chyme (**A**), caecal chyme (**B**) and their ratio (**C**) in rats 72 h after the single total body X-ray exposure at a dose of 9.64 Gy, M ± *m*, *n* = 8, depending on the duration of access to food since the time of irradiation. Control — non-exposed animals. Values of the group of non-exposed rats which had the unlimited access to food are at the zero mark of horizontal axis. * — significant difference from control, $p < 0.05$

the gastric chyme relative weight. Accumulation of chyme in the *caecum* was slower, which increased the gastic to caecal chyme weight ratio by 2–6 times depending on the duration of access to food relative to corresponding values of non-exposed animals (Fig. 2).

In exposed rats not receiving cystamine and deprived of food 24 h after irradiation, body weight was 78.9 ± 1.1% of the baseline value 72 h after exposure. In non-exposed animals deprived of food within the same time frame, it was 84.6 ± 0.7% of the baseline value ($p < 0.05$). Furthermore, the relative weight of gastric and caecal chyme in exposed rats was 5.9 and 2.3 times higher than that of intact rats, respectively. Cystamine administration before irradiation partially prevented gastric stasis: the relative weight of gastic chyme was on average three times lower than that of unprotected animals. The use of cystamine returned the gastic to caecal chyme weight ratio of 1.1 ± 0.2 in unprotected animals to the value of 0.4 ± 0.2 typical for intact rats, with equal duration of access to food ($p < 0.05$). Cystamine had no significant effect on the relative weight of caecal chyme in exposed rats. Cystamine administration also had little effect on the radiation-induced hypotrophy of the spleen (Fig. 3A). Urinary indican excretion measured 72 h after irradiation was on average twice higher than that of intact rats; cystamine had no significant effect on indicanuria (Fig. 3B). Indican excretion by the exposed rats receiving no radioprotector negatively correlated with the relative weight of gastric chyme, $r_s = -0.77$, and positively correlated with the relative weight of caecal chyme, $r_s = 0.68$ ($p < 0.05$); weak correlation was observed against the background of cystamine administration. Irradiation decreased the activity of enterocyte markers (ChE and ALP) in the small intestinal tissues. The most significant decrease (4.8-fold) in the ChE activity was observed in the *ileum*. The values of ChE activity in all parts of the small intestine and ALP activity in the duodenum and ileum tended to moderately exceed these values of unprotected rats against the background of using cystamine. This most prominent increase (2.5-fold) was reported for ChE in the ileum, however, it was represented in the form of the trend only (Fig. 3C and D).

The whole body oxygen consumption was lower than that of intact animals throughout the period after irradiation. On day three, this trend was significant when calculating per both time unit and respiratory cycle; the trend was stronger in the latter case. Intergroup differences in RR were insignificant. Cystamine administration had little effect on the gas exchange and external respiration characteristics (Fig. 4).

DISCUSSION

Modelling myeloablative radiation therapy in rats was associated with deep inhibition of the stomach propulsive function developing in the first hours after irradiation. The time of the chyme gastric transit exceeded two days, while the normal values of healthy humans do not exceed 48 min [21]. Extrapolation of these data to humans shows that gastric stasis

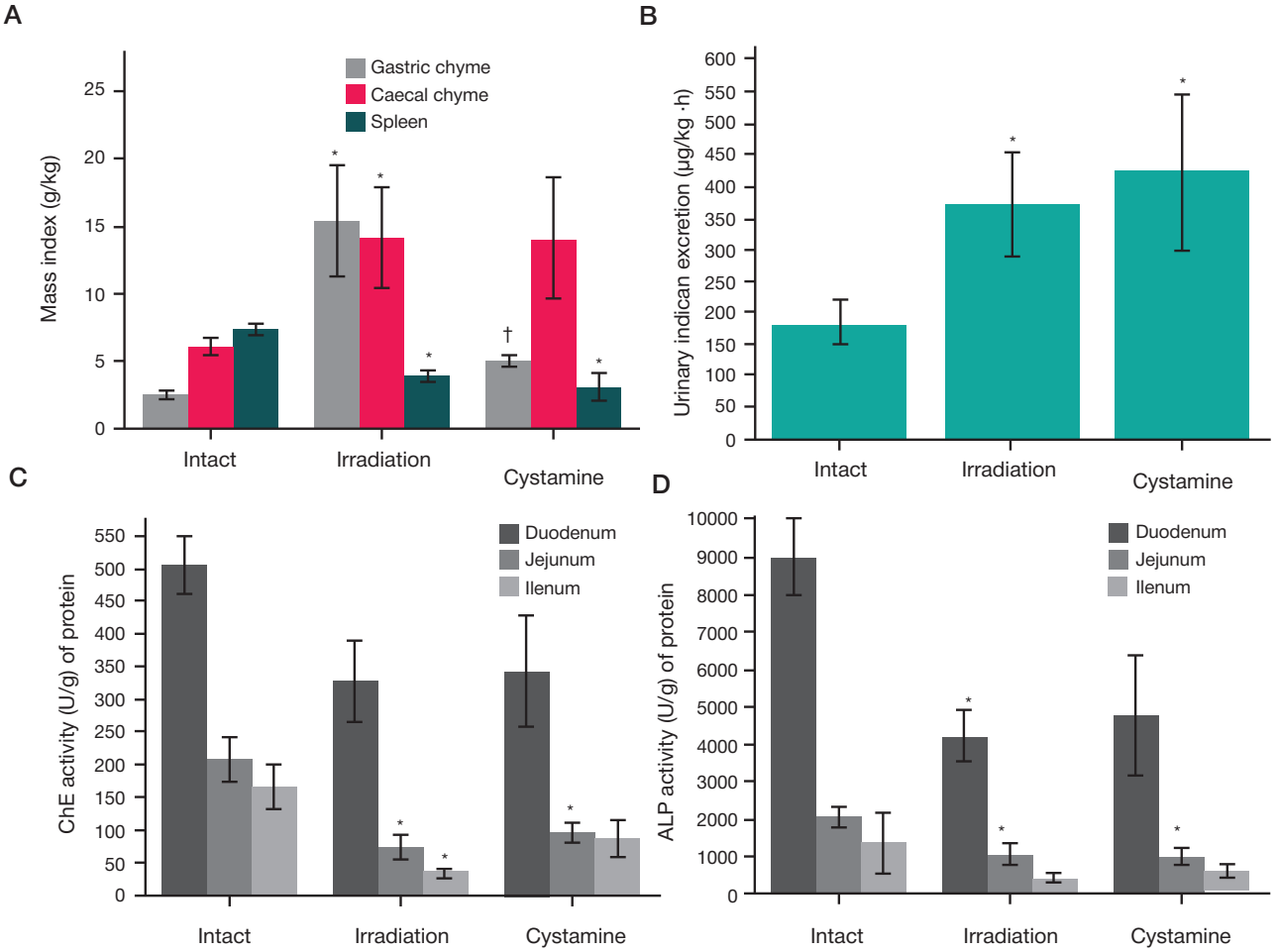

Fig. 3. Mass indexes of gastric chyme, caecal chyme and the spleen (**A**), urinary indican excretion (**B**), cholinesterase (**C**) and alkaline phosphatase (**D**) activity in the intestinal tissues of rats 72 h after the single total body X-ray exposure to the dose of 9.64 Gy, M ± *m*, *n* = 8, depending on the duration of access to food since the time of irradiation. "Intact" — non-exposed rats which obtained no medication. "Irradiation" — rats exposed without administration of radioprotector. "Cystamine" — intragastric administration of cystamine dihydrochloride in a dose of 120 mg/kg 30 min before the beginning of irradiation. All animals were deprived of food 24 h after irradiation. Significant difference, $p < 0.05$: * — from intact group; † — from "Irradiation" group

persists for most of the myeloablative conditioning course. It can be associated with the complaints typical for such patients: loss of appetite, nausea, vomiting, pain, epigastric heaviness and bloating. Rodents lack emesis relieving the stomach; that is why the stomach overfilling could be more prominent in rats, than in humans exposed to equal doses.

Despite inhibition of the chyme release into the caecum resulting from gastric stasis, the relative weight of caecal chyme measured 3 days after irradiation was 2.3 times higher than that of non-exposed animals, which reflected the decrease in the colonic propulsive function. The total relative weight of gastric and caecal chyme increased by 3.4 times in exposed rats: on average to 29.5 vs. 8.8 g/kg in controls. Despite accumulation of chyme, body weight after irradiation was 7% lower than in non-exposed animals fasting during the same time period, which indicates possible involvement of GI stasis in deterioration of body's general condition. Intestinal endotoxicosis, indicated by the two-fold increase in the urinary indican (indoxyl sulfate) excretion, could be one of the mechanisms underlying such an effect. Indoxyl sulfate is the end product of indole oxidation to indoxyl and its sulfonation in the liver. The reaction catalyzed by the gut microbiota-derived tryptophanase is the only source of indole in the body. Toxicity is exhibited by both indoxyl sulfate concentration two order of magnitude exceeding physiological levels [22] and indole [23]. Hyperindicanuria is indicative of more intense production of ammonia, the other toxic product of the tryptophanase reaction, in the gastrointestinal tract, along with indole. Endotoxicosis may involve other intestinal toxic substances and products of their biotransformation: bacterial endotoxin, *p*-cresol, *p*-cresyl sulfate, trimethylamine, trimethylamine N-oxide, influx of which into blood is increased when there is GI stasis [22].

The content of bacteria in the colonic chyme, 10^{11} mL^{-1}, is eight orders of magnitude higher than that in the gastric lumen, ≤ 10^{3} mL^{-1} [24]. That is why accumulation of chyme in the caecum played a major role in the development of intestinal endotoxicosis. Under these circumstances, gastric stasis that slowed chyme entry into the *caecum* could limit intestinal endotoxicosis. This is indicated by negative correlation between the relative weight of gastric chyme and the urinary indican excretion, as well as by positive correlation between the latter and the relative weight of caecal chyme in exposed rats not receiving radioprotector.

The gastric stasis protective role could come not only from its inhibiting effect on production of toxic substances in the intestine, but also from prevention of further damage to the small intestinal epithelium by chyme released from the stomach under conditions of emerging enterocytopenia. It was indicated by the decrease in the enterocyte marker (ChE and ALP) activity in the small intestinal tissues after irradiation.

A more than three-fold decrease in the relative weight of gastric chyme associated with intragastric administration of cystamine resulted from its local radioprotective effect on the gastric mucosa (Fig. 3A). This was evident from the lack of

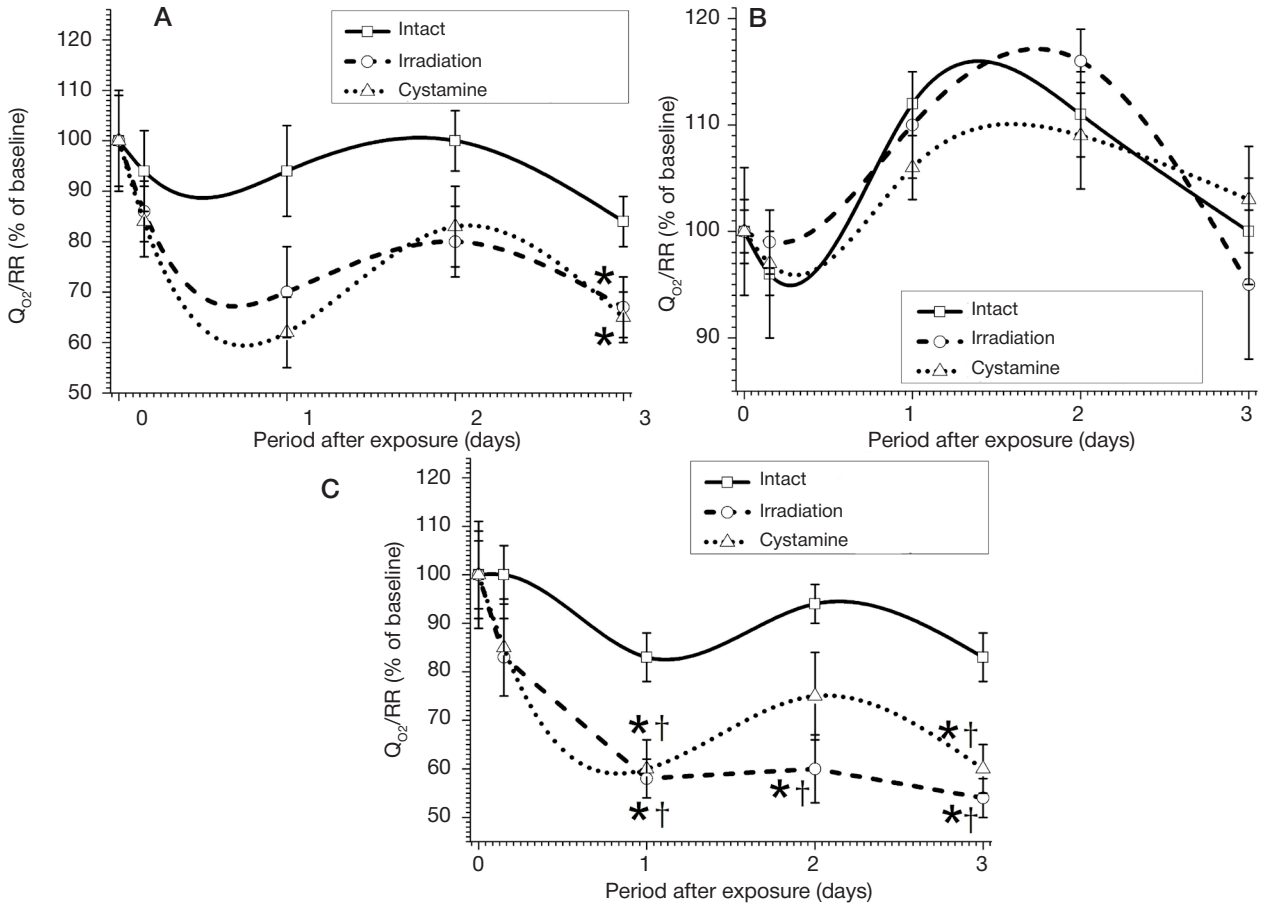

Fig. 4. The whole body oxygen consumption (**A**), respiratory rate (**B**) and oxygen consumption per respiratory cycle (**C**) in rats 72 after the single total body X-ray exposure at a dose of 9.64 Gy, M ± *m*, *n* = 8. Q_{O2} — the whole body oxygen consumption; RR — respiratory rate. 100% is the parameter values determined at 4 h before irradiation. * — significant difference from baseline, $p < 0.05$; † — significant difference from intact group, $p < 0.05$

significant cystamine effect on the radiation-induced spleen hypotrophy, the sensitive indicator of systemic radioprotector effects. Such a result is consistent with impossibility to reproduce the cystamine systemic radioprotective effect by itragastric cystamine administration to rats reported in the literature [19]. Cystamine prevented gastric stasis, despite its thiol form (cysteamine) capability of reversible inhibition of gastric chyme evacuation via enchanced hydrochloric acid secretion by the gastric parietal cells known from the literature [25].

Gastric stasis prevention could not be mediated by the cystamine local radioprotective effect in the small intestinal mucosa: the effect was weak, which was evident from the lack of significant influence on the enterocytopenia characteristics, ChE and ALP activity in the small intestinal tissues (Fig. 3C and D). It can be assumed that the anatomical structure of the rat stomach lead to the fact the radioprotector solution failed to enter the small intestine till the end of irradiation and contacted mostly with the gastric mucosa.

The hypothesis of gastric stasis prevention as a result of cystamine local radioprotective effect on the gastric mucosa is consistent with the emergence of GI stasis in rats after local irradiation of the abdomen reported more than 70 years ago, while irradiation with equal doses without abdominal shielding never causes GI stasis [10]. The findings suggest that the GI stasis triggers are localized in the mucous membranes of appropriate gastrointestinal tract parts and can be “switched off” through local exposure to cystamine.

In case of ingestion of equivalent cystamine dihydrochloride dose (1.2 g) by humans 30–40 min before radiation exposure to the dose causing bone marrow syndrome, the nominal radiation dose change factor is 1.4 [7]. This means that in case of ingestion of the recommended dose of cystamine by humans, gastric stasis prevention would be associated with systemic radioprotective effect that is unwelcome when preparing patients for hematopoietic stem cell transplantation. That is why selectivity of emergency gastric stasis prevention involving cystamine during exposure of rats to myeloablative doses cannot be unconditionally extrapolated to humans. Using cystamine to prepare patients for hematopoietic stem cell transplantation requires determination of the conditions for realization of its capability of preventing gastric stasis without exhibiting myeloprotective activity in large animals.

In this study we assessed gas exchange under conditions that were close to the conditions for determination of basal metabolic rate, that is why the oxygen consumption decrease observed in exposed animals could not result from their dazed state. The finding is consistent with the reduced oxygen consumption in rats earlier followed up for 3 days after the X-ray exposure to the doses of 300–1000 R [26]. Gas exchange inhibition could not result from reduction of respiratory volume due to restriction of diaphragmatic excursion by the dilated stomach: this was evident from the lack of significant irradiation effect on the RR (Fig. 4B). This also could not result from the direct damaging effect of the applied radiation dose on the tissue energy metabolism: there is no information about such effect in the literature. Reduced whole body oxygen consumption could be a manifestation of intestinal endotoxicosis indicated by the increased urinary indican excretion in exposed animals (Fig. 3B). Such gut microbiota products, as bacterial endotoxin and *p*-cresyl sulfate, are characterized by the capability of

causing damage to the blood-gas barrier followed by pulmonary edema [27, 28]. Indoxyl sulfate and bacterial endotoxin damage mitochondria, thereby disturbing oxygen utilization at the cellular level [29, 30]. The hypothesis of the intestinal endotoxicosis involvement in the effect of gas exchange reduction following radiation exposure is supported by no effect of cystamine on the latter; preventive cystamine administration did not prevent hyperindicanuria.

The data obtained show that the pathogenetic approach to prevention of gastric stasis caused by myeloablative radiation exposure involving the use of radioprotectors is promising. This approach cannot be considered as an alternative to using symptomatic drug treatment to relieve primary systemic response to irradiation (particularly, treatment with 5-HT_3 receptor antagonists).

CONCLUSIONS

The single total-body X-ray exposure of rats to the dose of 9.64 Gy corresponding to that used for myeloablative conditioning leads to the decrease in enterocyte counts in the small intestinal mucosa after 3 days, as well as to gastrointestinal stasis with predominant gastric stasis and hyperindicanuria being an indicator of excessive growth of intestinal microbiota producing indole. Intragastric administration of the cystamine dihydrochloride dose equivalent to that recommended as a single dose for humans to rats 30 min before irradiation partially prevents gastric stasis and has no significant influence on the characteristics of enterocytopenia, caecal stasis, as well as on the hyperindicanuria severity.

Modelling radiation-induced myeloablation in rats is associated with reduction of the animals' oxygen consumption not resulting from the influence of gastric stasis on the diaphragmatic excursion within 3 days after exposure. Intragastric administration of cystamine prior to irradiation does not prevent this effect. In rats, local radioprotective effect of cystamine dihydrochloride injected in the stomach is not associated with the emergence of the signs of systemic radioprotective effect, which, when repoduced in large laboratory animals, makes this drug a promising agent for prevention of gastric stasis during myeloablative radiation therapy.

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COMPUTATIONAL PHANTOM FOR A 5-YEAR OLD CHILD RED BONE MARROW DOSIMETRY DUE TO INCORPORATED BETA EMITTERS

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The red bone marrow (RBM) exposure due to bone-seeking radionuclides can lead to grave medical consequences. In particular, the increased risk of leukemia in people exposed due to contamination of the Techa River in 1950s is associated with the RBM exposure due to ^{89,90}Sr. Improvement of the internal RBM dosimetry methods includes the development of computational phantoms that represent 3D models of the skeletal sites. Modeling radiation transport within such phantoms enables estimation of conversion factors from the radionuclide activity in the bone to the RBM dose rate. This paper is an extension study focused on generating a set of computational phantoms representing skeletons of individuals of different ages. The aim was to develop a computational phantom representing a 5-year-old child for internal RBM dosimetry from incorporated beta emitters. The phantoms of the skeletal sites with active hematopoiesis were created using the original Stochastic Parametric Skeletal Dosimetry (SPSD) method. With this method, every such site represented a set of smaller phantoms of simple geometric shape. RBM distribution across the skeleton, bone size, characteristics of bone micro-architecture, as well as density and chemical composition of the simulated media (RBM, bone) were determined based on the published data. As a result, a computational phantom of the major skeletal sites with active hematopoiesis representing a 5-year-old child was generated that included 43 phantoms of bone fragments. Linear dimensions of phantoms were within 3–75 mm. Micro-architecture parameters varied greatly: *BV/TV* ratio —13–52%, *Tb. Th*. — 0.09–0.29 mm, *Tb. Sp*. —0.48–0.98 mm.

Keywords: trabecular bone, cortical bone, bone marrow dosimetry, computational phantoms, Sr

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ВЫЧИСЛИТЕЛЬНЫЙ ФАНТОМ ДЛЯ ДОЗИМЕТРИИ КРАСНОГО КОСТНОГО МОЗГА ПЯТИЛЕТНЕГО РЕБЕНКА ОТ ИНКОРПОРИРОВАННЫХ БЕТА-ИЗЛУЧАТЕЛЕЙ

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Облучение ККМ (красного костного мозга) остеотропными радионуклидами может приводить к серьезным медицинским последствиям. В частности, увеличение риска развития лейкозов у людей, подвергшихся радиационному воздействию в результате загрязнения реки Течи в 1950-е гг., связано с облучением ККМ от ^{89,90}Sr. Совершенствование методов внутренней дозиметрии ККМ включает разработку вычислительных фантомов, которые представляют собой трехмерные модели участков скелета. Моделирование переноса излучений внутри таких фантомов позволяет оценить коэффициенты перехода от активности радионуклида в кости к мощности дозы в ККМ. Настоящая статья — продолжение работы по созданию набора вычислительных фантомов скелета для людей разного возраста. Цель: разработать вычислительный фантом скелета пятилетнего ребенка для внутренней дозиметрии ККМ от инкорпорированных бета-излучателей. Фантомы участков скелета с активным гемопоэзом создавали с использованием оригинальной методики SPSD (stochastic parametric skeletal dosimetry). В рамках этой методики каждый такой участок представлял собой набор меньших фантомов простой геометрической формы. Распределение ККМ в скелете, размеры костей, характеристики костной микроархитектуры, а также плотность и химический состав моделируемых сред (ККМ, кость) определяли на основе опубликованных данных. В результате был сгенерирован вычислительный фантом основных участков скелета с активным гемопоэзом для пятилетнего ребенка, включающий 43 фантома участков костей. Линейные размеры фантомов были в пределах от 3 мм до 75 мм. Параметры микроархитектуры варьировали в широких пределах: отношение *BV/TV* — от 13% до 52%, *Tb. Th.* — от 0,09 мм до 0,29 мм, *Tb. Sp*. — от 0,48 мм до 0,98 мм.

Ключевые слова: трабекулярная кость, кортикальная кость, дозиметрия костного мозга, вычислительные фантомы, Sr

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After entering the body, bone-seeking radionuclides accumulate in the mineralized bone tissue and cause local red bone marrow (RBM) exposure, which can lead to grave medical consequences. Thus, for example, the increased risk of leukemia and the development of chronic radiation syndrome in people from the cohort of the Techa River contaminated with radionuclides in 1950s are largely attributed to ingestion of strontium isotopes (^{89,90}Sr) [1–4]. It was strontium isotopes that were the main sources of the RBM internal exposure in these people. Thus, improvement of dosimetry methods for

bone-seeking radionuclides can help prepare for potential emergency situations related to radioactive contamination of the environment and represents an important challenge of radiobiology and radiation protection. Biokinetic and dosimetric models are used to estimate the absorbed dose to RBM. Biokinetic ones are used to assess specific radionuclide activity in the source tissue (skeletal bones). Such models simulate metabolic processes in the body allowing one to estimate the ingested radionuclide fraction in various organs, particularly in the bones, depending on its quantity and time after ingestion [5]. The dose conversion factors (DF) for conversion of specific radionuclide activity in the source tissue (skeleton) into the absorbed dose rate in the target tissue (RBM) are used to calculate the dose to RBM. DF represents a dosimetric model output. The computational skeletal dosimetry phantoms representing surrogates of real body tissues and describing relative positions of the source (bone) and target (RBM) tissues, in which radiation transport is simulated, are used to simulate the exposure geometry. The existing computational phantoms for RBM dosimetry are based on the analysis of a limited number of post mortem bone CT images [6–12]. The use of such phantoms makes it impossible to consider individual variability in bone size and microstructure, as well as the associated uncertainty in the DF estimates. As an alternative, Stochastic Parametric Skeletal Dosimetry (SPSD) modeling, the original parametric method for stochastic bone structure modeling, was developed in the Urals Research Center for Radiation Medicine [13, 14]. The SPSD phantom parameters are based on the numerous published measurement results of real bones in people of different ages. A lot of statistics reported in the papers used enable estimation of uncertainties associated with individual variability in the skeletal parameters. A phantom is a virtual model of simple geometric shape. A computational phantom is filled with spongiosa (a combination of trabecular bone and bone marrow) and covered with a dense layer of cortical bone. RBM, trabecular bone and cortical bone were modeled as distinct media constituting the phantom. Such complex model is a simplified representation of the real bone that is well suited for internal dosimetry from the bone-seeking beta emitters [13, 14]. The model consistency was demonstrated in the reported numerical experiments [13, 15, 16] yielding the energy curves for SPSD phantoms that were matched to the published data.

When the environment is contaminated with bone-seeking radionuclides (e.g. contamination of the Techa River), these can be ingested by people of different ages (from newborns to adults) [1–3, 17]. That is why it is important to develop phantoms for various age groups. We have already created phantoms representing skeletons of a newborn [18] and a 1-year-old child [19] within the framework of the SPSD approach.

The study was aimed to develop a computational phantom representing a 5-year-old child's skeleton for estimation of doses to RBM from the beta emitting radionuclides incorporated in the bone. This study represents the next stage of work on the development of a set of reference man computational phantoms for various age groups.

METHODS

Phantoms were created using the original SPSD method previously used to create phantoms representing a newborn [17] and a 1-year-old child [18].

Only the bone fragments containing RBM, i.e. skeletal sites with active hematopoiesis (hematopoietic sites), determined in accordance with the published data on the RBM distribution across the skeleton, were modeled within the framework of the SPSD methodology [20].

The SPSD phantom of the skeletal hematopoietic sites consists of a set of smaller phantoms, the Bone Phantom Segments (BPS) of a simple geometric shape with homogenous bone tissue microarchitecture and cortical layer thickness, describing distinct skeletal bone sites. Such segmentation simplifies the modeling process and enables estimation of the bone microarchitecture heterogeneity within a single hematopoietic site. BPS parameters are based on the published data. The segmentation process details are provided in the earlier reports [13, 21].

Parameters of phantoms included the mineralized bone tissue and bone marrow (simulated media) chemical composition and density, along with the geometry of the source and target tissues comprised in the modeled bone fragment.

Chemical composition and density of the simulated media determined based on the published data were used in all phantoms representing a 5-year-old child [22, 23].

To describe relative position and geometry of tissues inside the bone, we assessed linear dimensions, cortical layer thickness (*Ct. Th.*), and microarchitecture characteristics for each modeled bone site.

To assess morphometric parameters of the phantoms representing a 5-year-old child, we reviewed articles published in peer-reviewed journals, atlases, manuals, monographs, and dissertations. We also reviewed digital resources containing collections of x-ray images. To perform analysis, we collected the measurement results of individuals/samples that were considered to be healthy by the authors and had no disorders resulting in bone deformities. Ethnicity: Caucasians and Mongoloids, since these groups are typical of the Urals region. The subjects' age was 3–7 years.

In this study, the following bone microarchitecture characteristics were assessed based on the published data: trabecular thickness (*Tb. Th.*), trabecular separation (*Tb. Sp.*), bone fraction in the spongiosa volume (*BV/TV*). We considered the measurement data of skeletal bones obtained using various techniques: micrometers, osteometric board, ultrasound and radiography, as well as computed tomography (CT). Histomorphometry and micro-CT data were used to estimate the trabecular bone parameters (*Tb. Th.*, *Tb. Sp.*, *BV/TV*) and the cortical layer thickness.

Average estimates of bone characteristics were taken as computational phantom parameters. When the published data on individual measurements were available, we combined these data to calculate the means and standard deviations (SD). When the measurement results of groups of people were averaged, a weighting factor (W_N) for each group considering the number of subjects (N) was introduced: $W_N = 1$, when $N \geq 25$; $W_N = N/25$, when $N < 25$. Methods to select and assess the published data were previously discussed in detail in [24–26].

A voxel BPS was constructed for each segment using the original Trabecula software [27]. The BPS voxels imitated either mineralized bone, or bone marrow (BM), depending on the voxel center position in the phantom.

Trabecular bone (TB) and cortical bone (CB) were considered as source tissues, while bone marrow (BM) was considered as target tissue. BM was evenly distributed across the trabeculae in the BPS. Voxel size selected individually for each phantom did not exceed 70% of trabecular thickness and varied between 50–200 μm in the generated phantoms [27, 28]. The source and target tissue volumes were automatically calculated in the Trabecula software for each BPS.

Hematopoietic sites of a 5-year-old child, segmentation and BPS generated are provided in Fig. (exemplified by the scapula).

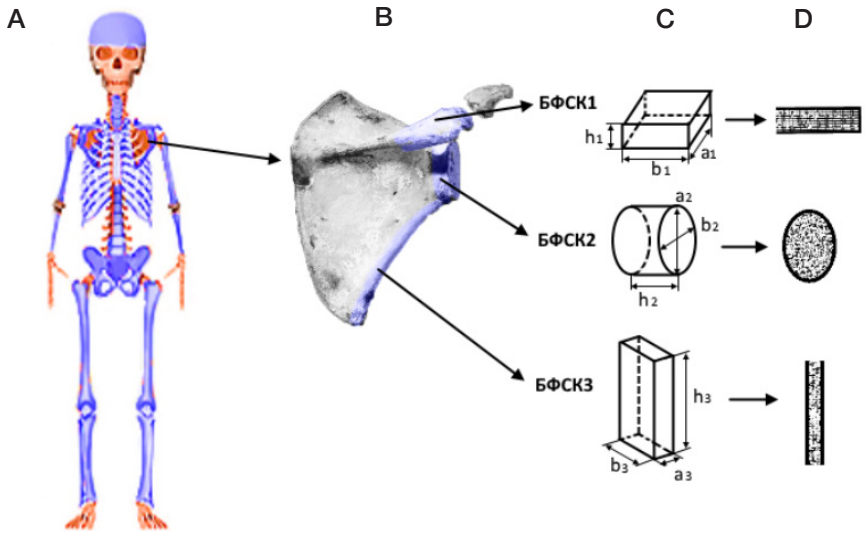

Fig. Segmentation of the skeletal hematopoietic site of a 5-year-old child exemplified by the scapula. **A**. Skeleton of a 5-year-old child (skeletal sites with active hematopoiesis are highlighted in *blue*). **B**. Scapula. **C**. Scheme of bone division into BPS and BPS dimensions. **G**. BPS of the scapula — voxel representation, cross section (voxels simulating bone tissue are highlighted in *black*, those simulating RBM are highlighted in *white*)

To simulate population variability in the size and micro-structure characteristics for each BPS, 12 Supplementary Phantom Segments (SPS) were created with the bone micro- and macro-structure parameters randomly selected within the range of their individual variability (within the limits of minimum and maximum measured values).

RESULTS

The main hematopoietic sites of the 5-year-old child's skeleton and the mass fraction of RBM in these sites were determined based on the MRI data (Table 1) [20].

The skeleton of a 5-year-old child includes 14 hematopoietic sites for simulation (Table 1). The share of RBM in these sites in the total skeletal RBM content varies from 0.9% to 18.1%. Distribution of RBM within each hematopoietic site was also determined based on the published MRI data [29–33].

Chemical composition of the simulated media was selected based on ICRP data for adults (Table 2) [22].

The mineralized bone tissue density estimated based on the cortical bone density measured in 5-year-old children was 1.80 g/cm^3 [23]. The RBM density was taken equal to the water density (1 g/cm^3) [22].

The spongiosa parameters were assessed based on the published data; the analysis and calculation of average population value of the spongiosa parameters were described in detail in [26]. The BPS micro-architecture parameter values for a 1-year-old child are provided in Table 3.

The linear dimensions and thickness of the cortical layer assumed/used for the BPS representing a 5-year-old child are provided in Table 4.

The phantom representing skeletal hematopoietic sites of a 5-year-old child consists of 43 BPS (Table 4). A different number of BPS was used to describe these, depending on the shape of the simulated hematopoietic site: 1 (ribs) to 9 (sacrum).

As in phantoms representing skeletons of people of other ages, most BPS of a 5-year-old child are cylinders and rectangular parallelepipeds; BSP size is within 3–75 mm. The minimum *Ct. Th*. value was reported for the sternal BPS (0.1 mm), while the maximum value was reported for the femoral diaphysis (3.7 mm). The spongiosa parameters varied greatly. The *BV/TV* ratio of BPS was 13–52%, *Tb. Th*. was 0.09–0.29 mm, and *Tb. Sp.* was 0.48–0.98 mm (Table 3).

The average individual variability of the BPS dimensional parameters was 14%; the highest variability value was reported for the ribs (35%), while the lowest value was reported for the

Table 1. Mass fraction of RBM (% of the total mass of RBM in the skeleton) in the main hematopoietic sites of the 5-year-old child's skeleton

№	Hematopoietic site	RBM mass fraction, %
1	Femur	13.5
2	Humerus	4.8
3	Sacrum	5.7
4	Tibia bones	9.3
5	Pelvic bones	13.5
6	Skull	18.0
7	Clavicle	0.9
8	Scapula	2.8
9	Sternum	1.7
10	Ribs	9.0
11	Radius and ulna	2.1
12	Cervical vertebrae	2.3
13	Thoracic vertebrae	9.2
14	Lumbar vertebrae	7.0

Table 2. Chemical composition of simulated media adopted for all BPS

Chemical composition, rel. units		
Chemical element	Bone	Active marrow
H	0.035	0.105
C	0.16	0.414
N	0.042	0.034
O	0.445	0.439
Na	0.003	0.001
Mg	0.002	0.002
P	0.095	0.002
S	0.003	0.002
Ca	0.215	–

distal part of the humerus (3%). The average cortical layer thickness was 26%, with the maximum value of 35% reported for the sacral segments and the minimum value of 7% reported for bodies of the cervical vertebrae. The average variability of spongiosa parameters was 18%, with the minimum value of 2% and maximum value of 70%.

Variability values were used for SPS modeling. SPS volume varies greatly, it can differ from the BPS volume by a factor of three, both upwards and downwards. In our future studies, DFs will be calculated for both BPS and SPS. Standard deviation of DFs calculated for SPS from DFs calculated for BPS will characterize the DF population variability.

DISCUSSION

Comparison of the modeling outcome with the masses of real bones was performed for the newborn in order to confirm the methodological approach accuracy. We have found no masses or volumes of the wet bones of 5-year-old children, that is why we cannot compare the modeling outcome with the real bones. The existing computational phantoms obtained by scanning autopsy material are non-parametric, that is why it is impossible to compare parameters of these phantoms with the SPSD phantom parameters. For most bones, it is also impossible to compare the characteristics of phantoms obtained as a result of their generation (masses of simulated media) with the published phantom masses, since only skeletal sites with active hematopoiesis are modeled within the framework of the SPSD approach, not the entire skeleton. The SPSD phantom masses (sums of masses of all segments comprised in the phantom) were compared with the published data for the bones, which were modeled as a whole: pelvic bones and the clavicle of a 5-year-old child [7, 12]. The comparison showed that the difference between the phantom masses reported in the published papers and the SPSD phantom masses did not exceed 20%.

It makes sense to assess age-related changes in the characteristics of phantoms representing a newborn, 1-year-old and 5-year-old children. The phantom representing a skeleton of a 5-year-old child consists of a larger number of BPS than the phantoms representing skeletons of younger children. This is associated with the processes underlying cartilage mineralization that are intense within the first five years of life; in particular, vertebral processes and the sternum have undergone significant mineralization by the age of 5. Furthermore, the process of the RBM substitution with adipose tissue in the tubular bone diaphysis is not over yet in children of this age, that is why BPS were modeled for these sites. Significant changes in the RBM distribution across the skeleton occur by the age of 5. Thus, children of this age have a significantly lower mass fraction of RBM in the skull compared to 1-year-old children (28.7% vs. 18.1%), furthermore, fraction of RBM in other skeletal segments is increased. The BPS micro-structure parameters show little changes. The cortical layer thickness

Table 3. Spongiosa parameters taken for BPS of a 5-year-old-child [11, 34–55] (coefficient of variation (CV) is given in parentheses, %)

Hematopoietic site	*BV*/*TV*, %	*Tb. Th*, mm	*Tb. Sp*, mm
Femur (proximal part)	35 (6)	0.24 (22)	0.77 (70)
Femur (central and distal parts)	26 (6)		
Humerus	22 (7)	0.21 (13)	0.58 (47)
Ribs	20 (6)	0.23 (34)	0.51 (14)
Tibia bones*	25 (3)	0.13 (13)	0.74 (11)
Pelvic bones	25 (2)	0.15 (11)	0.48 (23)
Skull*	52 (5)	0.29 (31)	0.57 (35)
Clavicle*	15 (3) 29 (9)	0.2 (32) 0.15 (13)	0.80 (25)
Radius and ulna	16 (5)	0.16 (13)	0.77 (16)
Scapula*	22 (8)	0.24 (42)	0.96 (23)
Sternum*	15 (27)	0.14 (33)	1.0 (6)
Cervical vertebrae	21 (5)	0.14 (14)	0.60 (20)
Thoracic vertebrae + lumbar vertebrae + sacrum	13 (4)	0.09 (40)	0.60 (20)

Note: * — spongiosa parameters were calculated based on the measurement results of similar bones or based on the data for other age groups; the calculation method was reported previously in [25].

Table 4. Linear dimensions and thickness of the cortical layer taken for the BPS representing a 5-year-old child

Hematopoietic site	Segment	Shape[1]	Phantom parameters, mm (CV is given in parentheses, %)[2]						Data sources
			h	*a*	*b*	*c*	*d*	*Ct.Th.*	
Femur	Diaphysis[4]	c	30	17 (6)	17 (6)			3.7 (8)	[56–64]
	Proximal end (upper part)	c	25 (4)	23 (30)	23 (30)			1.3 (14)	
	Proximal end (lower part)	c	25 (4)	23 (30)	23 (30)			1.2 (14)	
	Distal end	dc	49 (4)	68 (6)	25 (7)	17 (6)	17 (6)	1.1 (7)	
Humerus	Diaphysis[4]	c	30	15 (3)	15 (3)			2.5 (20)	[56–59,65]
	Proximal end	dc	20 (4)	32 (5)	32 (5)	15 (5)	15 (5)	0.9 (18)	
	Distal end	dc	27 (4)	46 (7)	15 (3)	15 (3)	15 (3)	0.8 (19)	
Ribs	Ribs[4]	p	9.4 (32)	30	4.4 (35)			0.5 (33)	[66–68]
Sacrum	Body of the 1st vertebra	p	17 (20)	75 (20)	21 (6)			0.7 (35)	[69–72]
	Body of the 2nd vertebra	p	16 (20)	60 (20)	15 (10)			0.7 (35)	
	Body of the 3rd vertebra	p	14 (20)	52 (20)	11 (10)			0.7 (35)	
	Body of the 4th vertebra	p	10 (20)	45 (20)	6.4 (9)			0.7 (35)	
	Body of the 5th vertebra	p	10 (20)	22 (20)	6.4 (9)			0.7 (35)	
Tibia bones	Fibula[4]	c	30	8.1 (6)	8.1 (6)			1.5 (20)	[56, 73, 74]
	Tibia diaphysis[4]	c	30	15(4)	15 (4)			2.9 (17)	[55, 56, 60, 75–77]
	Tibia proximal end б. б.	dc	34 (5)	55 (6)	22 (20)	15 (4)	15 (4)	0.7 (18)	
	Tibia distal end	dc	34 (5)	24 (22)	24 (23)	15 (4)	15 (4)	0.7 (18)	
Pelvic bones	Ilium part 1	p	7.9 (13)	30	30			"1.6 (33) 0.8 (20)3"	[78–85]
	Ilium part 2	p	7.9 (13)	30	30			0.8 (20)	
	Acetabular part of the ilium	dc	20 (8)	35 (10)	16 (30)	34 (30)	27 (30)	0.8 (20)	
	Acetabular part of the pubis	dc	7.3 (15)	22 (20)	18 (20)	13 (11)	8.8 (20)	0.5 (30)	
	Pubis bone (superior ramus)	c	29 (15)	13 (11)	8.8 (20)			0.5 (30)	
	Pubis bone (inferior ramus)	c	19 (15)	8.8 (20)	8.8 (20)			0.5 (30)	
	Acetabular part of the ischium	p	21 (15)	21 (15)	27 (15)	21 (15)		0.5 (30)	
	Ischial tuberosity	c	25 (15)	18 (15)	14 (15)			0.5 (30)	
	Inferior ramus of the ischium	c	19 (15)	8.8 (20)	8.8 (20)			0.5 (30)	
Skull	Flat bones[4]	p	4.2 (26)	30	30			1.1 (26)	[86–88]
Clavicle	Body[4]	c	30	8.7 (9)	6.8 (10)			1.1 (9)	[89–92]
	Sternal end	dc	12 (13)	18 (10)	16 (9)	8.7 (9)	6.8 (10)	0.5 (10)	
	Acromial end	dc	12 (13)	15 (11)	8.7 (18)	8.7 (9)	6.8 (10)	0.5 (10)	
Radius and ulna	Diaphysis[4]	c	30	8.3 (25)	8.3 (17)			1.5 (12)	[56, 58, 73]
	End	dc	26 (5)	13 (6)	8.3 (5)	8.3 (5)	8.3 (5)	0.5 (29)	
Scapula	Glenoid	c	12 (8)	25 (11)	18 (7)			0.9 (28)	[93–96]
	Acromion	p	7.6 (18)	20 (12)	16 (12)			0.8 (13)	
	Lateral border	p	30	3.2 (6)	10 (12)			0.8 (13)	
Sternum	Sternum	p	6.9 (13)	30	30			0.1 (19)	[37, 97, 98]
Cervical vertebrae	Vertebral body	c	7.3 (8)	11.3 (12)	18.1 (12)			0.2 (7)	[51,99–102]
Thoracic vertebrae	Vertebral body	c	12 (17)	17 (17)	21 (20)			0.2 (25)	[51,100–103,104]
	Transverse process	p	7.3 (19)	11 (19)	5.3 (19)			0.2 (25)	
	Spinous process	p	5.9 (21)	17 (21)	3 (21)			0.2 (25)	
Lumbar vertebrae	Vertebral body	c	16 (11)	23 (12)	34 (13)			0.2 (25)	[51,71,100–103, 105]
	Transverse process	p	6.4 (13)	12 (12)	5 (12)			0.2 (25)	
	Spinous process	p	15 (20)	13 (20)	5 (20)			0.2 (25)	

Note: [1] — phantom shape was designated as follows: c — cylinder, dc — deformed cylinder, *p* — rectangular parallelepiped, e — ellipsoid; 2 — BPS dimensions were designated as follows: *h* — height; *a* — major axis (c), major axis for a larger base (dc) or side a (p); *b* — minor axis (c), minor axis for a large base (dc) or side b (p); *c* — major axis for a small base (dc); *d* — minor axis for a small base (dc); [3] — cortical layer thickness was considered to be different for the inner (medial) and outer (gluteal) surfaces of this segment of the ilium; [4] — BPS imitated only a part of the simulated bone segment, when the bone segment dimensions significantly exceeded 30 mm, since in such cases it makes no sense to simulate the entire bone fragment in terms of dosimetry [14, 21].

Table 5. Comparison of BPS volumes of 1-year-old and 5-year-old children

BPS	Simulated medium	Modeled structure volume, cm^3		
		1 year	5 years	1 year / 5 years
Distal end of the femur	BM	6.53	22.9	3.51
	TB	1.88	7.56	4.02
	CB	1.41	5.21	3.7
	Entire BPS	9.82	35.67	3.63
Sternal end of the clavicle	BM	0.35	0.89	2.54
	TB	0.14	0.36	2.57
	CB	0.09	0.22	2.44
	Entire BPS	0.58	1.47	2.53
Body of the lumbar vertebra	BM	1.32	8.51	6.45
	TB	0.2	1.34	6.7
	CB	–	0.3	–
	Entire BPS	1.52	10.15	6.68
Body of the cervical vertebra	BM	0.45	0.89	1.98
	TB	0.11	0.24	2.18
	CB	–	0.05	–
	Entire BPS	0.56	1.18	2.11

significantly increases during this period; all the modeled skeletal segments become covered with the cortical layer by the age of 5, in contrast to phantoms representing a newborn and a 1-year-old child. In the period from the age of one to five years, the size of all parts of the skeleton increases significantly. Comparison of the volumes of phantoms representing skeletal segments of 1-year-old and 5-year-old children exemplified by the distal part of the femur, clavicle, bodies of the cervical and lumbar vertebrae is provided in Table 5.

As shown in Table 5, the volumes of simulated media of a 5-year-old child largely exceed those of the 1-year-old child. The increase in the source tissue volume for this period is 3.26-fold and 2.78-fold for TB and CB, respectively. The CB volume increase is 3.03-fold. The total BPS volume of a 5-year-old child is on average 2.8 times larger than the volume of the phantom representing a 1-year-old child.

We expect that such age-related changes will have a significant impact on the average DF of the skeleton and, therefore, on the dose rate. The increase in the BPS linear dimensions can have the greatest effect on the DF for strontium incorporated in the trabecular bone. Previous studies have shown that the larger the BPS size, the greater the chance is of absorbing energy from the incorporated radionuclide within the phantom, not outside it [15, 16]. The opposite pattern is typical of strontium in the cortical bone: the larger the phantom size, the lower the likelihood of energy transfer from the source incorporated on the outer cortical layer to the target (RBM) is. Thus, when the BPS size increases, one can expect the increase in DF for ^{89,90}Sr in the trabecular bone and decrease in DF for Sr in the cortical bone.

The phantom parameters provided (Tables 3, 4) can be integrated in the Trabecula software to generate voxel phantoms. Modeling of radiation transport using voxel phantoms will make it possible to assess DFs for bone-seeking beta emitters, thereby allowing one to determine the RBM absorbed dose rate.

CONCLUSIONS

The study resulted in the development of computational phantoms representing the main skeletal sites of a 5-year-old child with active hematopoiesis. These phantoms were developed using the same method, as for the newborn and 1-year-old child. The phantoms modeled imitate bone tissue structure, while the sets of phantoms simulate population variability in the size of structures of distinct bones. The provided phantom representing a 5-year-old child will be used to calculate DFs for ^{89,90}Sr, which, in turn, are essential for estimates of adjusted coefficients linking individual radionuclide uptake and dose to RBM, which will help to improve dose estimates for the Urals region residents. As the way forward, we also plan to create SPSD phantoms representing skeletons of people of other age groups: 10 year-olds, 15-year-olds and adults. SPSD phantoms can be used for the incorporated bone-seeking beta emitter dosimetry in the population, in case of radionuclide contamination of the environment, as well as for dosimetry from other bone-seeking beta emitters, including those used in radionuclide therapy, such as ^{89}Sr, ^{32}P, ^{186}Re, ^{188}Re, ^{117}mSn.

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THE ROLE OF FAST RUNNING IN PREVENTION OF NEGATIVE EFFECTS OF PROLONGED EXPOSURE TO WEIGHTLESSNESS

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The prospects of deep space exploration necessitate modification of the principles and methods underlying the system designed to prevent negative impact of weightlessness on the human body. This work aimed to determine how fast running, as part of locomotor training during a space flight (SF), helps maintain physical ability of a person. The study involved 10 cosmonauts; their physical performance was assessed at all stages of the SF with the help of the Individual Strategies Test (IST). The parameters registered when the participants were doing the IST included heart rate (HR), gas exchange, capillary blood lactate concentration. The cosmonauts were divided into two groups based on the differences in the mean distance covered while fast running on a treadmill (single session). Group A (n = 4) run 949 m/day on average, group B (n = 6) — 2669 m/day. After SF, HR in group A increased at speeds from 5 to 8 km/h ($p < 0.05$), pulmonary ventilation indicators grew at speeds from 8 to 15 km/h ($p < 0.05$), and the capillary blood lactate concentration measured during the post-test recovery period increased by 37% (p = 0.03). Moreover, after SF, the pulse sum recorded under load and during recovery was 14% (p = 0.02) and 15% (p = 0.03) in group A, respectively, while in group B we registered no differences. Thus, our hypothesis that fast running triggers sensory reactions simulatingEarth conditions for the body, which consequently activates physiological mechanisms counteracting the negative effects of weightlessness, has been confirmed in a space experiment.

Keywords: locomotor training, physical activity test, physical performance, space flight, ergospirometry

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Author contribution: Fomina EV — organization and support of the Profilactika-2 experiment, conducting sessions of the experiment, article authoring; Senatorova NA — conducting sessions of the experiment, support of the experiment, statistical processing of the results, literary review and arrangement of the article; Bakhtereva VD — data processing, article authoring; Yarmanova EN — engineering support of countermeasures, development of the BD-2 treadmill in collaboration with I.B. Kozlovskaya; Kozlovskaya IB — selection/formulation of goals, objectives and methods of the experiment.

Compliance with ethical standards: the Profilactika-2 experiment was approved by the Ethics Committee of the Institute of Biomedical Problems (Minutes #368 of August 22, 2014). All participants signed a voluntary informed consent form.

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РОЛЬ БЫСТРОГО БЕГА В ПРЕДОТВРАЩЕНИИ НЕГАТИВНЫХ ВЛИЯНИЙ ПРЕБЫВАНИЯ ЧЕЛОВЕКА В НЕВЕСОМОСТИ

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Перспектива освоения дальнего космоса определяет необходимость модификации принципов и методов системы профилактики негативного влияния невесомости на организм человека. Целью исследования было определить роль бега с высокой скоростью во время локомоторных тренировок, выполняемых в ходе космического полета (КП), в сохранении уровня физической работоспособности человека. В исследовании приняли участие 10 космонавтов. Оценка физической работоспособности проводилась на всех этапах КП на основе теста «Индивидуальные стратегии» (ТИС). Во время выполнения ТИС регистрировались частота сердечных сокращений (ЧСС), параметры газообмена, концентрация лактата в капиллярной крови. Космонавты были разделены на две группы на основе различий в среднем объеме бега с высокой скоростью в ходе одной тренировки на дорожке. В группе А (n = 4) средняя дистанция быстрого бега составила 949 м/день, в группе Б (n = 6) — 2669 м/день. ЧСС в группе А после КП увеличилась на ступенях от 5 до 8 км/ч ($p < 0,05$). Повышение легочной вентиляции после КП наблюдалось в группе А на ступенях нагрузки от 8 до 15 км/ч ($p < 0,05$). После КП концентрация лактата в капиллярной крови в периоде восстановления после теста в группе А увеличилась на 37% (p = 0,03). Пульсовая сумма работы и восстановления оказались выше после КП в группе А на 14% (p = 0,02) и 15% (p = 0,03) соответственно, в то время как в группе Б различий не обнаружено. Таким образом, наша гипотеза о том, что бег с высокой скоростью воспроизводит сенсорный приток, сопоставимый с условиями Земли, и, как следствие, обеспечивает включение физиологических механизмов, противодействующих негативному влиянию невесомости, подтверждена в космическом эксперименте.

Ключевые слова: локомоторные тренировки, тест с физической нагрузкой, физическая работоспособность, космический полет, эргоспирометрия

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Development of methods of preservation of health and physical ability of cosmonauts during long space flights is a key task for space medicine [1–4]. Preparation for Moon and Mars missions, or survival scenarios in the event the ship lands in an unplanned place, substantiate the quest for ways to maintain high levels of cosmonauts' performance , to support functional reserves and reliability of their bodies, to ensure effectiveness of their actions when discharging complex extravehicular tasks on the surface. Prolonged exposure to weightlessness affects cardiovascular [5–10], respiratory [11], and musculoskeletal [12–14] systems; thus, prevention of the negative effects thereof should be aimed to all of them. Data collected during space flights and from simulations indicate that in axial unloading, translates into sensory deprivation degrading regulation of the support afferentation, which subsequently leads to atony, muscle fiber atrophy, and compromises the vestibular system [14, 15]. Proprioceptive and tactile inputs enable postural control, therefore, activating the respective systems and keeping them "tuned" to maintaining vertical balance with the help of running in zero gravity can help improve the ability to perform functional tasks after the flight [16, 17]. Thus, intensive physical training designed to counteract the weightlessness-induced negative changes in the functioning of gravity-dependent physiological systems is a mandatory component of medical support during long space flights [16, 18–20].

Previously, we determined the values of axial load and the volume of locomotion needed to move treadmill in the passive mode using leg strength, which translates into an effective locomotor training during space flight [21]. This study aimed to assess the role of fast running on a moving treadmill in the context of locomotor training effectiveness. In our opinion, countermeasures to the negative impact of weightlessness can take form of the work that simulates keeping weight elevated or moving it in the conditions of the Earth. It can be said that the preventive efficacy of the method revolves around mechanical work that creates conditions reproducing the effects of gravity and generates the respective sensory inputs. In weightlessness and with lack of mechanical loading, only intensive exercising activates metabolic and functional systems to the levels comparable to those specific to Earth conditions. The purpose of this study was to determine the role of fast running in maintaining a person's physical performance during a long-term space flight.

METHODS

Characteristics of the examined individuals

The article presents results of the Profilaktika-2 experiment conducted during a space flight. The study involved 10 cosmonauts (age 44 ± 6 years, weight 84 ± 6 kg, duration of space flights 173 ± 33 days). The inclusion criteria were gender (male), and space flight duration (about 6 months). The exclusion criteria were incomplete or untimely completion of the experiment sessions, and significant deviations from training protocols designed for the space flight.

Prevention of negative effects of weightlessness during space flight

The method for prevention of the negative effects of weightlessness relies mainly on physical training. During the space flight, they consumed 2.5 hours a day on average, including preparations and hygienic procedures. According to the onboard documentation, the cosmonauts did two physical training sessions every day. BD-2 treadmill (Institute of Biomedical Problems; Russia) was used on a daily basis, and VB-3M ergocycle (Institute of Biomedical Problems; Russia) and ARED exercise device (NASA; USA) were alternated every other day.

Treadmill sessions are a key element of the countermeasures against hypogravity disorders developed for Russian cosmonauts. The on-board treadmill training protocols for a four-day microcycle were compiled based on the simulations run in the Earth conditions [22]. After introduction of the BD-2 treadmill, they were modified slightly, but still suggested alternating intervals of high-intensity running and walking. Strictly speaking, in accordance with the existing classification of training methods, all exercises under the protocols imply a pattern that alternates different maximum running speeds and physical exercise levels conditioned by the proportion of time when the treadmill is in passive-mode, i.e., it is rotated only by the strength of the cosmonaut's legs. BD-2 treadmill can work in active mode, i.e., it is driven by a motor, and in passive mode, when it is rotated by strength of the cosmonaut's legs.

All treadmill training protocols prescribed a warm-up of 4 minutes, which is running at 7 km/h, then — physical loading with the treadmill in passive mode, which is walking and two 2-minute sessions of running at 6-8 km/h.

The final component included 2 minutes of walking at 5 km/h, then running for 2 minutes at 8 km/h with treadmill in the active mode, then walking in the passive mode for 1 minute.

The main partof the treadmill training protocol changed on different days of the microcycle:

Day one — four 1-minute fast running takes (at 14 km/h), alternating with 2-minute walks.

Day two — two 2-minute passive mode running takes (at 8 km/h), one active mode running take (at 12 km/h), alternating with 2-minute walks.

Day three — 4-minute running takes (at up to 13 km/h), alternating with 2-minute walks.

There were no exercises prescribed for day 4, except for individual locomotor training.

Most of the participants followed the above-described protocols in their prevention activities; on the fourth day, four cosmonauts did not abstain from physical activity but started the microcycle anew, two cosmonauts worked out under individual protocols (interval training), and two more preferred to rest. Two cosmonauts who were in space for the fourth time followed a individuallocomotor training program that spanned 7 days, with the last day being Sunday, a day of rest.

The parameters of each training session during the long-term SF were analyzed based on the weekly ergometric and physiological data, the analysis yielding further treadmill training recommendations. The response of the cardiovascular system to locomotor loads was registered as reflected in the heart rate (HR) recorded during training. When the flight was over, we calculated the mean values of each type of locomotion, monthly and overall (entire flight). Among the participating cosmonauts, the main parameters of treadmill training — the magnitude of axial load, the ratio of passive and active treadmill modes, the distance covered in a day — varied only slightly. The recommended axial load value was 70% of the body weight or more, and, for the most part, the participants took this recommendation into account. As for the modes, the share of passive mode varied through the microcycle and amounted to 30% over three days. One cosmonaut, who followed an individual training program, run with the treadmill in passive mode only for 8.2% of the total daily training time, while for

all the other participants this value ranged from 23 to 41%. The distance covered during a session also varied through the microcycle and ranged from 3000 to 6000 m on different days.

For the strength training part enabled by ARED, all the cosmonauts had individual protocols. Initially, the load factored in the cosmonaut's body weight before flight, and during the flight it was adjusted to make the training process wavelike. Specialists supervising the program of countermeasures against negative effects of microgravity received information about exercises on ARED every week, and adjusted the workout routines.

As reported by the crew, they trained on the ergocycle following guidance from the on-board documentation, that is, alternating intervals of various intensity. Currently, there are no systems enabling transmission of objective information about the magnitudes of loads and the response of cardiovascular system to physical exercise on the ergocycle.

Experimental groups

The cosmonauts were divided into two groups based on the duration of fast running intervals. Pre-flight, locomotor tests revealed no differences between the groups.

During the flight, in group A (n = 4), the average distance covered while running fast, with treadmill in the active mode, was 949 m per day, while group B (n = 6) covered 2669 m per day under similar conditions.

Test procedures

The cosmonauts' physical performance was assessed on the basis of the IST 30 days before the flight, 3–4 times during the flight (42–68, 83–113, 115–131 and 140–156 days thereof), and 10 ± 2 days after its completion [23]. The IST followed a standard protocol and employed the BD-2 treadmill in active mode; the components thereof were a warm-up with alternating intervals of walking at 3 km/h and 6 km/h in a pseudo-randomized sequence, and an interval of growing load, from 3 km/h to 15 km/h, with the speed increasing for 1 km/h every 30 seconds.

During the test, heart rate was recorded using Polar (Polar; Finland) and Cardiocassette-2010 (Institute of Biomedical Problems; Russia). Ergospirometry (Earth conditions) was enabled by Oxycon Mobile (Jaeger; Germany), "breath-by-breath" method. The lactate content in capillary blood was measured using the Lactate-2 kit (Institute of Biomedical Problems; Russia), at rest before the test, then at the first and fifth minutes of the post-test recovery period.

The functional reserves of the cardiovascular system were assessed based on the total heart rate under load (area under the heart rate curve for the entire IST) and that during the recovery period (area under the heart rate curve reflecting the 5 minutes of recovery after the test). The said totals were sums of the heart rate values, which were registered every 10 seconds during the test and through the 5 minutes of the subsequent recovery.

The "heart rate deficiency" value was calculated as the difference between the number of recovery period heartbeats and that peculiar to relative rest [24]. This indicator reflects the post-exercise physiological and metabolic changes in the body. We also calculated the delta heart rate that shows the difference between maximum heart rate and resting heart rate.

Statistical data processing was performed using Minitab 19.1 (USA); it included checking the distribution in samples with the help of the Shapiro–Wilk test, calculating indicator means and variance (one-way ANOVA). The results were considered significant at $p < 0.05$ under the Fisher test or the Tukey test. We considered only the significant differences in the results.

RESULTS

Before the space flight, the groups were similar in all the studied indicators. Compared to the pre-flight data, heart rate increased significantly in group A at each load increment from 5 km/h to 8 km/h post flight. No such changes were registered in group B (Fig. 1). The post-flight IST did not reveal differences in heart rate between groups A and B.

In group A, compared to the pre-flight values, we registered a significant growth of pulmonary ventilation at each load increment from 8 km/h to 15 km/h (Fig. 2). In group B, this parameter was higher than before the flight only at the load increments of 9 km/h and 10 km/h. Compared at each load increment, the groups exhibited no differences in terms of pulmonary ventilation post-flight.

Comparing the respective pre-flight and post-flight data, we registered increased capillary blood lactate concentration

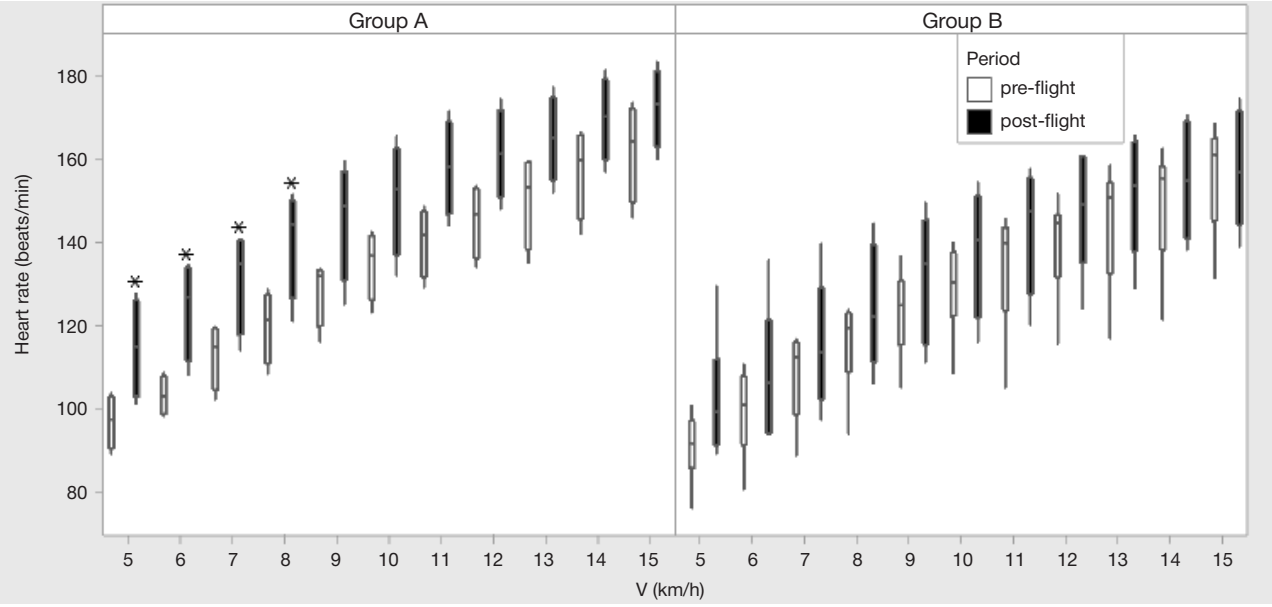

Fig. 1. Heart rate as registered with the IST before and after the space flight. * — compared to the preflight level in the group, $p < 0.05$

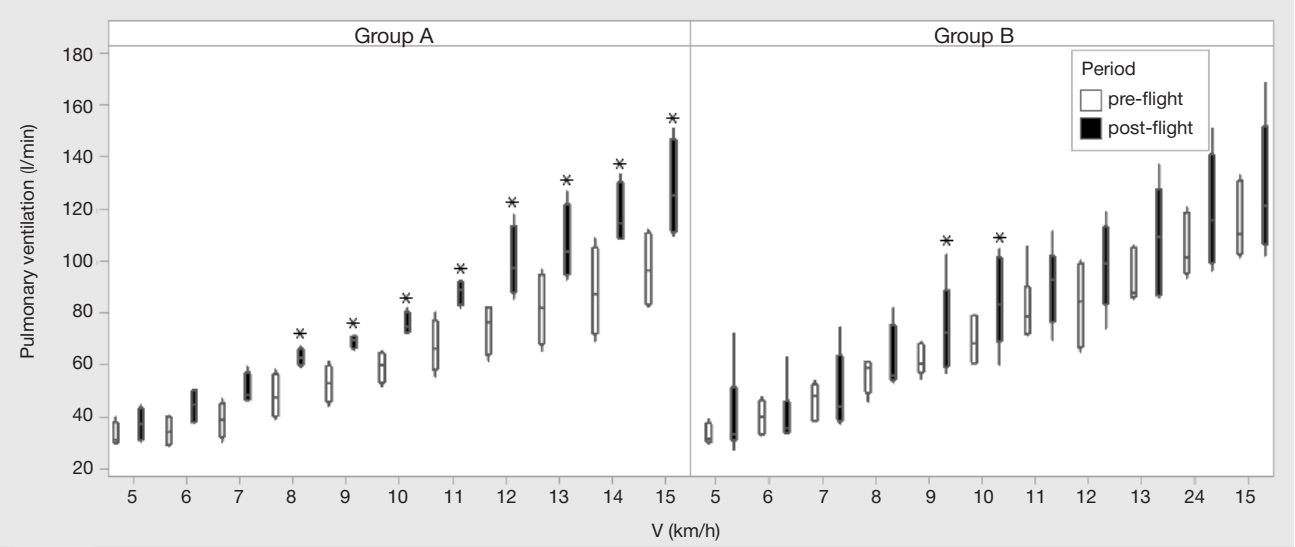

Fig. 2. Pulmonary ventilation before and after the space flight, as registered with the IST. * — compared to the preflight level in the group, $p < 0.05$

during the first minute of recovery (5.3 ± 1.6 before flight and 8.5 ± 3.4 mmol/L after flight, $p = 0.03$) in group A, and in group B these values did not differ significantly (5.3 ± 2.7 before flight and 6.7 ± 3.4 mmol/l after flight). We believe that higher capillary blood lactate concentration indicates flaws in utilization of this metabolite during exercise in group A, which means their level of physical ability was lower after the flight (Fig. 3).

At the final stage of the flight (days 140–156), we registered intergroup differences in the total heart rate values: they were 19.4% (17,897 ± 529) higher in group A than in group B (14,678 ± 3148) ($p = 0.009$).

Post-flight, the total heart rate value in group A was higher than the background value recorded before the space mission: 16,475 ± 1257 and 19,143 ± 1972, respectively ($p = 0.02$). In group B, the differences in this indicator were insignificant: 14,983 ± 1572 before the flight and 16,148 ± 2651 after the flight (Fig. 4).

At the final stage of the flight, the total recovery heart rate value was higher in group A than in group B: 2838 ± 188 and 2181 ± 490, respectively ($p = 0.009$) (Fig. 5).

Post-flight, the total recovery heart rate value in group A was 3027 ± 405, which is higher than what was registered in this group before the flight (2575 ± 326, $p = 0.03$) and more than seen in group B after the flight (2599 ± 350, $p = 0.02$).

Analysis of the heart rate deficiency, oxygen consumption, carbon dioxide release and maximum respiratory rate before and after the flight revealed no significant differences between the groups and within them.

DISCUSSION

We hypothesized that the effectiveness of prevention of the negative effects of weightlessness depends on the degree of reproduction of action of gravity. If the preventive measures bring around internal and external sensory inputs comparable to those peculiar to the Earth conditions, the body's gravity-dependent systems function nearly as if it had weight. The respective effects are reproduced most accurately when a cosmonaut is running on a treadmill in the special training suit that simulates 60-70% of his Earth body weight, exerting the load along the vertical axis. A person standing or performing locomotions on a treadmill works out, but the intensity of this training in space flight conditions is significantly lower than on Earth, mainly because the magnitude of axial load usually does

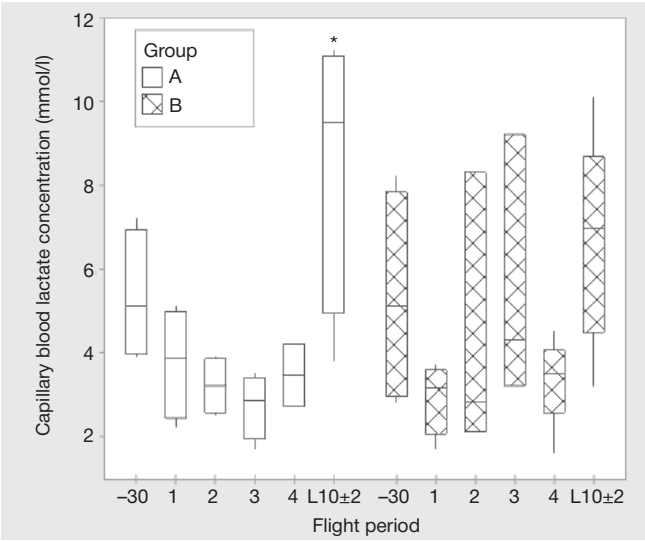

Fig. 3. Capillary blood lactate concentration, first minute of recovery after the IST. * — compared to the pre-flight level in the group, $p < 0.05$; 1 — days 42–68, 2 — days 83–113, 3 — days 115–131, and 4 — days 140–156

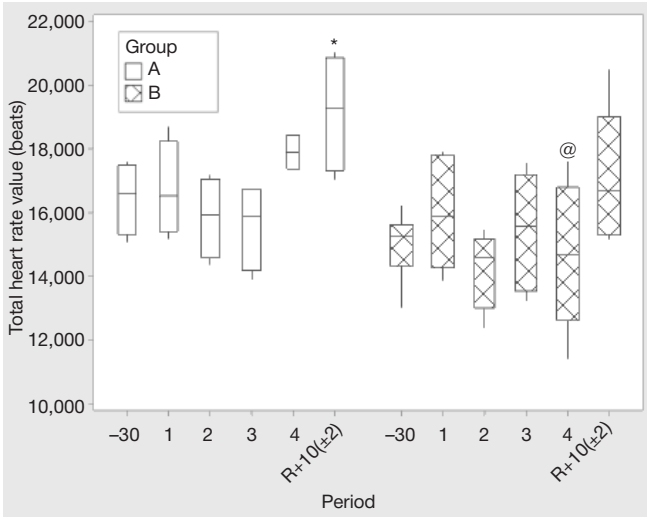

Fig. 4. Total heart rate value registered during the IST. * — $p < 0.05$ compared to the background value in the group; @ — $p < 0.05$ in comparison with the value of the same flight period in group A; 1 — days 42–68, 2 — days 83–113, 3 — days 115–131, and 4 — days 140–156 of the flight

not exceed 70% of that person's body weight on Earth. We have previously shown that running at the speed of 7 km/h in space triggers response from the body's support systems consistent with its weight at 1G [21]. Thus, in order to launch the physiological mechanisms governing muscular activity, and to have the respective vegetative system functioning in the mode resembling that peculiar to the Earth conditions, it is necessary to run at high speed. Obviously, the greater the physical load, the higher the physiological load. If a person's body is not only held upright, countering gravity simulated with the training suit, but also moves, then the physiological load increases, and the preventive effect is more pronounced. Based on the above, it was suggested that the proportion of fast running will play a role in maintaining the cosmonauts' physical performance after a long exposure to weightlessness.

Earlier, we have shown that alternating intensity in a training session (intervals of fast running and walking) is more effective from the prevention standpoint than running at a constant speed [25], the result consistent with a study that involved cosmonauts [26] and the present study, which registered a higher level of capillary blood lactate concentration at the 1st recovery minute after a locomotor load in the group that had fewer fast running intervals during the space flight. The mentioned higher lactate concentration indicates that the subject is in an unstable metabolic state that shifts the acid-base balance, which may be associated disruptions of operation of nerve centers, lower level of activity of enzyme systems, and, consequentially, inhibition of muscles [27–29]. In the group that had more fast running spans in the protocols, concentration of lactate in the capillary blood during the post-test recovery period was at the pre-flight level, which, according to the concepts of sports physiology, means healthy functioning of the aerobic mechanisms supplying energy to muscles, and retention of the ability to utilize lactate [30]. Protocols with a greater proportion of fast running launched physiological mechanisms that preserve the aerobic system supplying energy to the muscles, and, as a result, anaerobic mechanisms were triggered at the later stages of the incremental loading test. Accordingly, there was no significant accumulation of lactate, which is a product of the glycolytic system supporting muscle activity.

Increased pulmonary ventilation registered at days 10 ± 2 post-flight during the test at increments from 8 km/h to 15 km/h, compared to the pre-flight data, accords with higher capillary blood lactate concentration and indicates an overstrain of the oxygen transport system in the group that had fewer fast running intervals during the space flight. Other studies have also reported increased pulmonary ventilation registered with an ergocycle test in astronauts on the 10th day after a long-term space flight [18, 31].

The results of this study clarify the concepts of mechanisms countering the negative effects of weightlessness. Fast transition from low- to high-intensity locomotor training activates the vegetative systems supporting muscle activity, which underpins the efficacy of the alternating load method described previously [25]. In this study, we factored in only the distance traveled at a speed of more than 9 km/h, and the transitions from low-intensity to high-intensity activity were disregarded. We assume that fast running with an axial load of about 70% of the Earth body weight effectively prevents the adverse influence of weightlessness, since this load, in terms of energy consumption and sensory inputs, is comparable with maintaining the body in an upright position or walking slowly under the Earth's gravity. Thus, running at a speed of more than 9 km/h, as we believe, triggers gravity-dependent physiological mechanisms by simulating the Earth weight conditions.

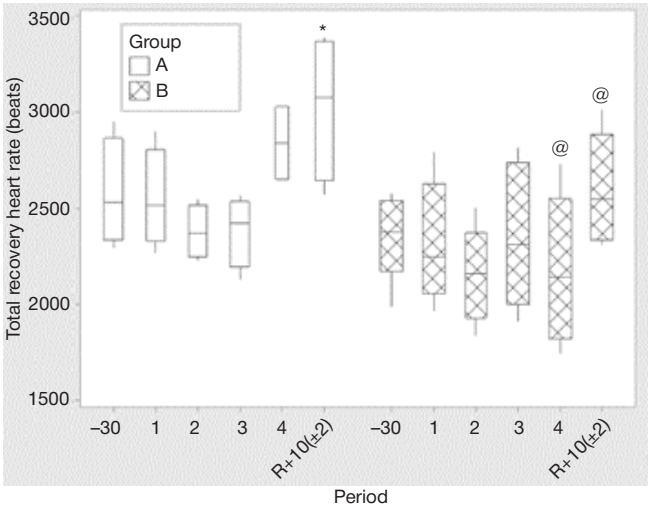

Fig. 5. Total recovery heart rate value registered during the IST. * — $p < 0.05$ compared to the background value in the group; @ — $p < 0.05$ in comparison with the value of the respective flight period in group A; 1 — days 42–68, 2 — days 83–113, 3 — days 115–131, and 4 — days 140–156 of the flight

There is a number of obvious limitations to a comparative analysis of the efficacy of countermeasures against adverse effects of weightlessness. It is possible to conduct a retrospective analysis of the effectiveness of training machines that were used earlier; such knowledge valuable, since some of them are still kept in the ISS as backups [32]. Earth-side simulations reproducing some effects of a space flight offer more extensive opportunities. One of such simulations is antiorthostatic hypokinesia (ANOG), which implies a supine position with the subject's head tilted down by 6°; ANOG simulations yield data on prevention of the negative effects of hypokinesia [33, 34]. A 90-day ANOG simulation experiment has shown that the most effective training program includes treadmill sessions with a vertical axial load of about 80% of the body weight and 80-90% of the maximum oxygen consumption, combined with high-intensity resistance training [34], which is consistent with results of our space experiment.

Our study identified new prognostic indicators in the IST, namely, the total heart rate value under load and the total recovery heart rate value; when these values were high during the space flight, the cosmonaut's physical ability post-flight was hindered. Countermeasures against negative effects of weightlessness will be more effective if these indicators are taken into account in the context of training supervision. In the interests of deep space exploration missions, it is planned to conduct studies investigating readaptation to Earth conditions in the earlier post-flight period and identifying the degree of applicability of the prevention methods used for an orbital flight.

CONCLUSIONS

Countermeasures against negative effects of prolonged exposure to weightlessness may be more effective if the cosmonaut has more sessions of fast running (speed of 9 km/h or faster).

The standard incrementally increasing locomotor load with the treadmill in active mode that allows registering the cardiorespiratory system's parameters provides data enabling prediction of the level of physical ability post-flight.

New predictors of the cosmonaut's physical ability after a long-term space flight are suggested, namely, the total heart rate value under load and the total recovery heart rate value, as registered with the help of a standard incrementally increasing locomotor load test.

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SPECIFICS OF REACTION OF HUMAN CARDIOVASCULAR SYSTEM TO IMMERSION IN COLD WATER

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Winter swimming implies extreme cold stress, which can cause respiratory disorders, arrhythmias, and elevated blood pressure even in generally healthy people. Pre-training examinations for athletes practicing winter swimming should include additional criteria evaluating reaction of the cardiovascular system (CVS) to cold water. This study aimed to determine the risk of pathological abnormalities in the examined individuals exhibiting different CVS reactions to immersion in cold water. We assessed reactivity of CVS with the help of a cold-hypoxic test (CHT), following a previously developed algorithm. The subjects of the analysis were CVS reactions to CHT and physical data collected after swimming in cold water. The study involved 255 female and 205 male participants, all of them almost healthy, aged 18–25 years. They participated in testing in a laboratory setting. Poly-Spektr-8/E cardiograph was used to record ECGs, and GraphPad Prism 8 package for Windows 10 for statistical analysis. Findings: in highly reactive and reactive participants, CHT causes lengthening of the PQ interval, with its value in the initial state (IS) equal to 158 ± 7.2, and with CHT — 178 ± 9.1 ($p < 0.01$); in subjects of he paradoxical type, CHT, against the background of higher pulse, triggered increase of QTc, which in the IS was 405 ± 7.1, with CHT — 420 ± 7.5 ($p < 0.05$). As for blood pressure, on average, CHT made it grow, SBD by 17.4 ± 4.3 mmHg, DBP by 12.9 ± 3.1 mmHg ($p < 0.05$). Swimmers adapted to cold, when swimming in cold water, had QTc above normal in 50% of cases: e.g., if IS of QTc was 434 ± 24 s, after swimming it increased to 492 ± 25 s. After a 200 m swim at $t = 1.5–2$ °C, the average blood pressure in the group, compared to IS, increased, with SBD growing by 16.9 ± 3.1 mmHg, and DBP — by 12.3 ± 2.3 mmHg ($p < 0.05$). Having analyzed the data, we conclude that CHT can be the basis of additional criteria extending examinations for athletes seeking admittance to cold water swimming.

Keywords: winter swimming, respiratory system, cardiovascular system, autonomic regulation, cardiac arrhythmias, intracardiac conduction, peripheral vasospasm, hypertension

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Author contribution: Baranova TI — article conceptualization, authoring, general editing; Rybyakova TV — article conceptualization, general editing; Dmitrieva MO — search for and analysis of sources, statistical data processing; Anisimov DA — search for and analysis of sources, compilation of tables, preparation of figures; Tarasova MS — search for and analysis of sources, article authoring; Ogannisyan MG — selection of approaches to mathematical modeling and their optimization.

Compliance with the ethical standards: the study was conducted in accordance with the Helsinki Declaration and approved by the Human Research Ethics Committee (Minutes #40 of March 07, 2012). All subjects were familiarized with the test protocol and signed an agreement to participate therein.

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ОСОБЕННОСТИ РЕАКЦИИ СЕРДЕЧНО-СОСУДИСТОЙ СИСТЕМЫ ОРГАНИЗМА ЧЕЛОВЕКА НА ПОГРУЖЕНИЕ В ХОЛОДНУЮ ВОДУ

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Зимнее плавание отличается экстремальной холодовой нагрузкой, которая может вызывать нарушение дыхания, аритмии, повышение АД (артериального давления) даже у почти здоровых людей. Спортсменам зимнего плавания необходимы дополнительные критерии допуска к тренировкам, оценивающие реакцию сердечно-сосудистой системы (ССС) на холодную воду. Целью исследования было определить риск патологических отклонений у обследованных с различной реактивностью ССС на погружение в холодную воду. Реактивность ССС оценивали посредством пробы холодо-гипоксического воздействия (ХГВ) по разработанному ранее алгоритму. Проанализированы реакция ССС на пробу ХГВ и данные после заплывов в холодной воде. В лаборатории обследованы практически здоровые 255 женщины и 205 мужчин 18–25 лет. ЭКГ регистрировали на кардиоанализаторе «Поли-Спектр-8/Е». Для статистического анализа использовали пакет GraphPad Prism 8 для Windows 10. Установлено: при ХГВ у высокореактивных и реактивных обследованных удлиняется PQ-интервал: в исходном состоянии (ИС) 158 ± 7,2, при ХГВ — 178 ± 9,1($p < 0,01$); у испытуемых парадоксального типа при ХГВ на фоне увеличения ЧСС наблюдали увеличение QTc — в ИС 405 ± 7,1, при ХГВ — 420 ± 7,5 ($p < 0,05$). При ХГВ относительно ИС в среднем АД повышалось — САД на 17,4 ± 4,3 мм рт. ст., ДАД на 12,9 ± 3,1 мм рт. ст. ($p < 0,05$). При заплывах в холодной воде у адаптированных к холоду пловцов в 50% случаев QTc превышал норму, например, в ИС QTc — 434 ± 24, после заплыва — 492 ± 25 с. После заплыва на 200 м при $t = 1,5–2$ °С в среднем по группе АД повышалось по сравнению с ИС САД на 16,9 ± 3,1 мм рт. ст., ДАД на 12,3 ± 2,3 мм рт. ст. ($p < 0,05$). Проанализировав данные, пришли к выводу — на основе пробы ХГВ можно разработать специфические критерии допуска к занятиям холодовым плаванием.

Ключевые слова: зимнее плавание, дыхательная система, сердечно-сосудистая система, автономная регуляция, сердечные аритмии, внутрисердечное проведение, периферический вазоспазм, гипертензивный ответ

Финансирование: исследование проведено в рамках выполнения своих трудовых обязанностей по заданию СПбГУ.

Вклад авторов: Т. И. Баранова — разработка концепции статьи, написание текста, общее редактирование; Т. В. Рыбьякова — разработка концепции статьи, общее редактирование; М. О. Дмитриева — поиск и анализ источников, статистическая обработка данных; Д. А. Анисимов — поиск и анализ источников, составление таблиц, подготовка рисунков; М. С. Тарасова — поиск и анализ источников, написание текста; М. Г. Оганнисян — определение подходов к математическому моделированию и их оптимизация.

Соблюдение этических стандартов: исследование проведено в соответствии с Хельсинкской декларацией и одобрено этическим комитетом СПбГУ (протокол № 40 от 07 марта 2012 г.). Все испытуемые подписали добровольное информированное согласие на участие в исследовании.

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In 2022, winter swimming was included in the Russian National Register of Sports. Extreme cold stress is inherent in this activity, which makes it significantly different from classical and open water swimming. The said stress also requires the practicing athlete to have higher functional reserves that should be evaluated and controlled.

There are two body temperature maintaining mechanisms triggered upon immersion in cold water without special equipment, one boosting bodily heat production and another limiting heat release [1, 2]. Such an act also activates reflective defense mechanisms: controlled by the sympatho-adrenomedullary system, peripheral vessels spasm, blood flow in skin and superficial muscles slows down (thus the output of heat is restricted), liver releases glucose, fat depots mobilize fatty acids, brown adipose tissue starts generating heat [3–5]. When the face is in the water, the body triggers diving reflex to save oxygen, and parasympathetic stimuli from cold receptors (mainly in facial skin) running along the vagus nerve's cholinergic pathway to the heart's sinus node intensify. Heart rate slows down, since the said reflex has a strong arrhythmogenic effect [6-8]. Further aggravation of the cholinergic stimuli affecting airways can lead to bronchoconstriction, which entails the risk of lung ventilation impairment [9].

Given the above, it should be noted that swimming in cold and ice-cold water is a rather common activity, which does not always trigger adverse reactions on the part of the cardiovascular system [10]. We believe that the negative reactions of the body that threaten human life when immersed in cold water may stem from high reactivity of the efferent part of reflex response. There are several factors that can make said reactivity high; for example, beginner cold swimmers may experience it because their bodies are unaccustomed to extreme cold, and other reasons may be rooted in the peculiarities of vegetative regulation formed during postnatal development, as well as in a person's genetic makeup [11–15].

This article analyzes the results of the earlier studies investigating the effect of cold water on adaptive reflex reactions, and, thereupon, seeks to characterize individuals at risk of a negative (health- and/or life-threatening) response to cold water immersion on the part of cardiovascular system.

The purpose of this work was to identify the specifics of adaptive cardiovascular reactions and assess the possibility of pathological abnormalities in the individuals exhibiting different vegetative reactivity of cardiovascular system to cold-hypoxia test (CHT) and cold water immersion in the context of swimming.

METHODS

We have summarized two types of research activities, laboratory and field. The first part presents materials from laboratory studies that involved triggering of the diving reflex by immersion of face into cold water, the cold-hypoxia test (CHT). We assessed arrhythmogenicity of the said reflex in people with different reactivity of the cardiovascular system to a cold stimulus.

The second part gives results of field studies, which took form of relay and competitive swims in water of different temperatures (+7–9 °C, +16–17 °C, and +1.5–2.5 °C). The analyzed factors of the cardiovascular system's reaction to swimming in cold water are myocardial conduction, blood pressure (BP), and heart rate (HR).

The study was conducted in 2008-2023, on the basis of St. Petersburg University, in the Systemic Adaptivity Laboratory. The participants were 205 male and 255 female individuals, all practically healthy, aged 18–25 years, whose cardiovascular system exhibited no pronounced abnormalities. Those with a history of sinus node dysfunction, stage II hypertension, atrial fibrillation were excluded from the study.

According to the survey, 15% of the participants were smokers with an average experience of 4.3 ± 1.7 years. As a rule, we asked all subjects to abstain from smoking and coffee for at least 2 hours before the test, which usually took place in the morning, from 10 a.m. to 12 p.m.

Laboratory part of the study

For the test, the subjects lied down on a couch, back up, in the most relaxed state. The test simulating diving involved immersing face in water. The water temperature was +10 ± 2.2 °C, the temperature of air in the room +21 ± 2.3 °C. As a rule, there were three immersions, all after an unforced exhale. The participants were not allowed to hyperventilate before the immersions. The rest period between the immersions was 2 minutes; as a rule, this time was sufficient for pulse to restore to its original rate. For the first immersion, the participants held their breath until they started to feel uncomfortable, and for the following immersions — for as long as they could.

To register response of the cardiovascular system, we used electrocardiography (ECG), photoplethysmography (PPG), and continuous blood pressure monitoring before, during and after immersion, while the participants were recovering. Peripheral vessels blood filling was indirectly determined by the amplitude of systolic wave in finger phalanges (ACB, pm), vascular tone — by the time of its propagation (VPRV, s). PPG was recorded and analyzed with the help of REAN POLY 6/12 rheograph-polyanalyzer, modification 03, version Elite (Medikom-MTD; Russia). To continuously monitor blood pressure, we used Finometer® MIDI (Finapres Medical Systems; Netherlands). The device employed to register ECG was Poly-Spektr-8/E cardioanalyzer (Neurosoft; Russia).

To describe the nature of the heart's chronotropic function during CHT, we used the following indicators: latent time of development of reflex bradycardia — l, s; time of occurrence of the maximum cardiointerval during the test — *t*max, s, bradycardia aggravation rate — V, severity of bradycardia — S.B. Earlier studies present a detailed description of the types determination method [16]; based on these indicators, we distinguished four types of response: highly reactive, reactive, areactive, and paradoxical (Fig. 1).

To analyze the data, we used GraphPad Prism 8 package for Windows 10. Mann-Whitney and Kruskal–Wallis tests allowed establishing the significance of differences for unrelated variables and for related paired samples. To assess the significance of differences in samples with a normal distribution, we used Student's *t*-test and one-way ANOVA. Statistical significance was registered at $p < 0.05$. In small groups, we looked for significance of changes for each person, before and after the swim, with ECG registered for 5 minutes. After assessing normality of distribution, we applied Student's *t*-test.

Results

Analysis of cardiovascular system's response to CHT revealed a reflex-driven heart rate slowdown, narrowing of the peripheral vessels (Fig. 2, 3; Table 1), and growth of the blood pressure (Fig. 2, Table 1).

In the first series of laboratory tests, which involved 460 subjects, we investigated chronotropic reaction of the heart to

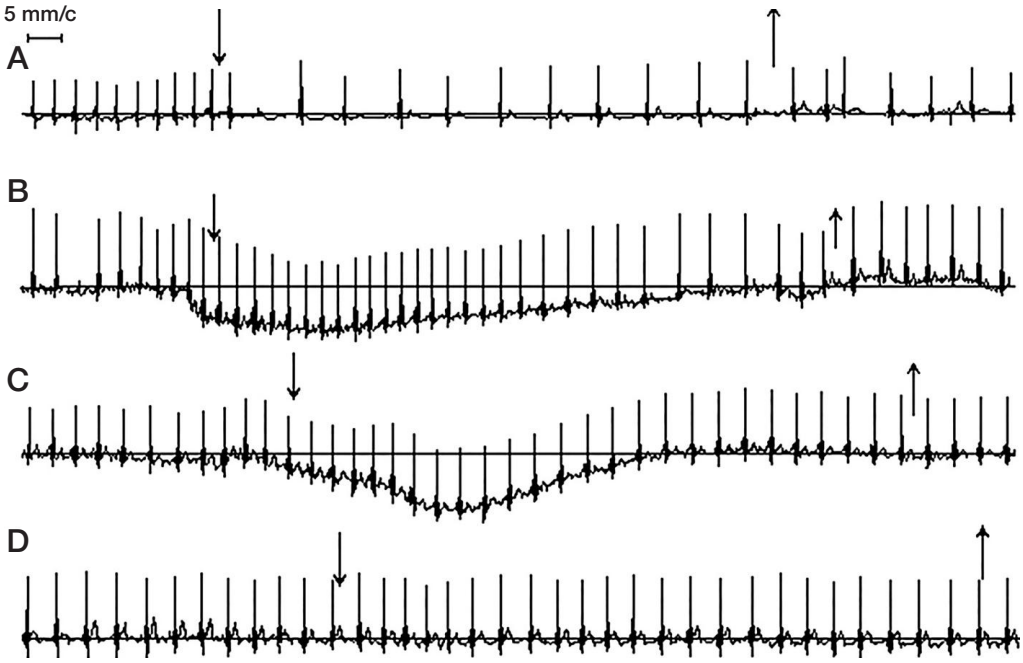

Fig. 1. Types cardiovascular system's response to immersion of face in water (types of diving reaction): highly reactive **(A)**, reactive **(B)**, areactive **(C)**, paradoxical **(D)**. Down arrow — immersion, up arrow — withdrawal from water

CHT. Based on the occurrence and rate of aggravation of reflex bradycardia (one of the diving reflex components) caused by CHT, the participants were distinguished into 4 types, according to the method we developed earlier [16]. The relative figures were as follows: 40% of the subjects belonged to the highly reactive type, 45% — to reactive type, 10% — areactive type, and 5% — paradoxical type. This typification by cardiovascular system's parasympathetic reaction to CHT was needed to understand whether it affects development of cardiac arrhythmias, and, conversely, what deviations from the normal can result in the participants from the paradoxical type group (predominance of sympathetic stimuli). This was necessary in order to assess the possible pathological abnormalities risk in people with various intensity of reflex parasympathetic and sympathetic stimuli sent to the myocardium when immersed in cold water.

Analysis of myocardial conduction during CHT in subjects with different types of reactivity

Analysis of the dynamics of changes of myocardial conduction indicators in response ot CHT revealed that in those of highly reactive and reactive type, reflex-driven parasympathetic stimuli not only slow down heart rate (statistically significant increases of RR intervals), but also alter the rate of atrioventricular conduction (PQ intervals increase). We observed CHT slowing down atrial conduction (PQ interval grows longer) in most participants belonging to the said type groups, but, as a rule, the registered PQ intervals in such cases were within the normal range. However, two individuals of the highly reactive type and three of the reactive type group had the PQ interval exceeding the norm, which indicates a delay in pulse conduction and partial atrioventricular blockade (Table 2).

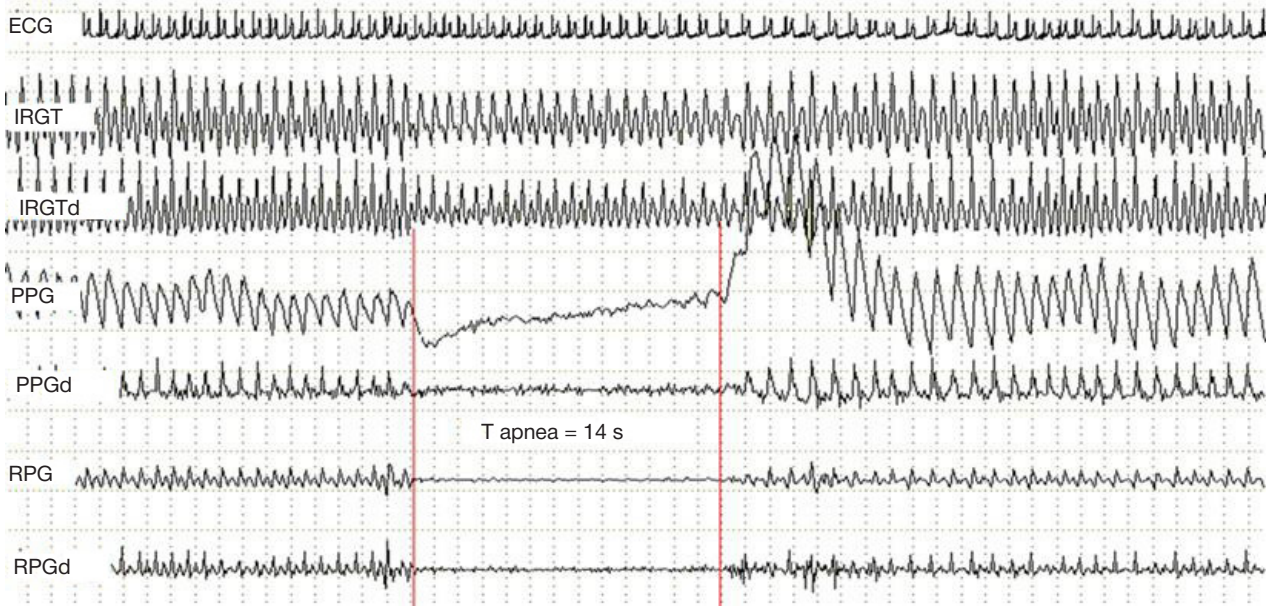

Fig. 2. Changes in the cardiovascular system characteristics during CHT. ECG — electrocardiogram, IRGT — Tishchenko integral rheogram, IRGTd — IRGT pulse ware differential curve, PPG — photoplethysmogram (distal phalanx of the thumb), PPGd — PPG pulse differential wave curve, RPG — shoulder rheoplethysmogram, RPGd — RPG pulse wave differential curve. Vertical lines — beginning and end of the test with face immersion in water

Table 1. Changes in the cardiovascular system characteristics at rest and during simulated diving, men and women (n = 80)

Indicators	HR, beats/minute	DBP, mmHg.	SBP, mmHg.	ACB, pm	VPRV, ms
At rest	64.8 ± 3.2	71.2 ± 5.8	113.8 ± 6.2	1.67 ± 0.91	217 ± 11.1
CHT	55.5 ± 3.8*	84.1 ± 7.3*	130 ± 10.1*	0.35 ± 0.20**	199 ± 20.5*
Recovery	63.5 ± 2.3	76.5 ± 5.1	121.1 ± 7.1	0.36 ± 0.19**	195.8 ± 15.3*

Note: SBP — systolic pressure, DBD — diastolic pressure, ACB photoplethysmogram pulse wave amplitude, VRPV — pulse wave propagation time. * — $p < 0.05$; ** — $p < 0.01$ — at comparison of the indicators during CHT and at the initial resting state.

In participants of areactive type, CHT triggered no significant changes (Table 4). In those of paradoxical type, against the background of CHT, RR, QT and TP intervals were decreasing, and QTC growing, which means that while HR accelerates, ventricular conduction is slowing down (Table 2).

Analysis of CHT-associated changes of blood pressure showed that DBP and SBP increased significantly in all the participants (Fig. 4, 5). We identified subjects of the highly reactive type to have higher initial BP. Those with paradoxical type had the lowest SBP. Moreover, in participants from the highly reactive group, repeated CHT caused DBP to progressively increase, reaching 175/115 mmHg in some cases.

Thus, in the majority of participants with moderate reactivity, CHT triggers reflex changes of adaptive nature, but in some subjects with high reactivity it can provoke atrioventricular blockade, cause a slowdown in ventricular conduction (in some of paradoxical type), push blood pressure up with a vasospasm in the background. These are the findings from CHT conducted in comfortable conditions with minimum stress. If the immersion in cold is full, the associated stress can make such deviations fatal.

Effect of cold-hypoxia training on reactivity of cardiovascular system

In order to establish the effect of adaptation to cold and hypoxia on vegetative regulation, we conducted a respective 6-week course with daily sessions. After CHT and typification by reactivity, 40 selected participants were distributed into 4 type groups (n = 10). Every day, in the course of 6 weeks, they immersed their faces into water with the temperature of +8 ± 2 °C, 3–4 times, having made an unforced exhale. After the course, CHT revealed decreased reactivity, which manifested mainly in a longer latent time of reflex bradycardia development and its slower progression. Seven individuals out of 10 from the highly reactive group transferred to the reactive group, but 3 retained reactivity at high level. From the reactive group, 4 participants moved to the areactive group, from paradoxical — 2 to the areactive, 3 to the reactive group, and 1 retained the original level of reactivity.

The analyses of reflex constriction of peripheral vessels and blood pressure dynamics also showed that training decreased

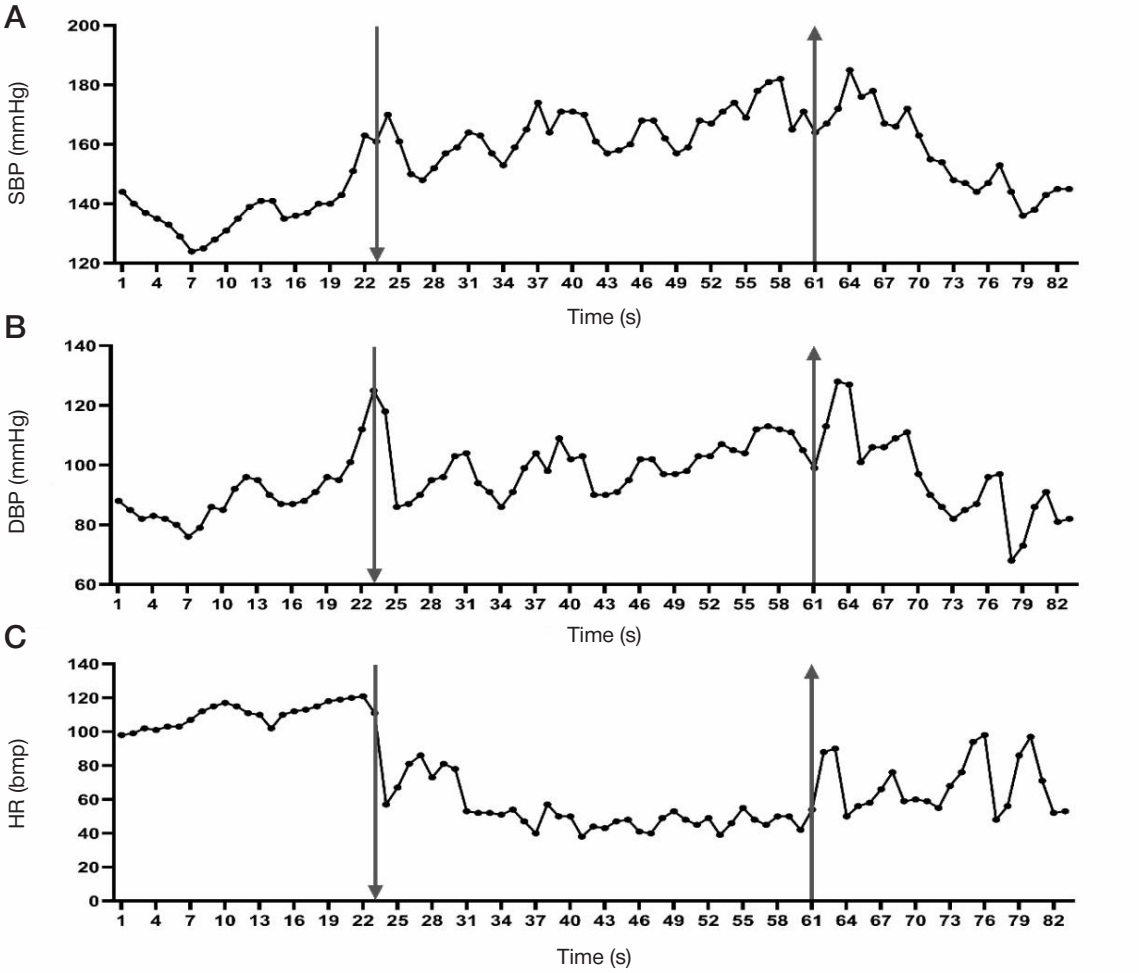

Fig. 3. Dynamics of blood pressure: systolic (SBP) (A), diastolic (DBP) (B), heart rate (HR) (C). Abscissa axis — time, ordinate axis for **A** and **B** — mmHg, for **C** — beats/minute. Down arrow — CHT begins, up arrow — CHT ends

Table 2. Temporal ECG indicators at rest and during CHT, participants of various types of reactivity

Indicators	Duration of cardiac cycle intervals, ms						
	RR av.	P	PQ	QRS	QT	QTc	TR
Highly reactive type (n = 18)							
At rest	1052 ± 22.7	98 ± 6.2	158 ± 3.9	97 ± 10.4	383 ± 6.7	418 ± 7.3	512 ± 20.2
During CHT	1466 ± 25.4**	99 ± 8.0	167 ± 4.1*	106 ± 8.6	403 ± 7.9	376 ± 11.3	906 ± 19.9**
Reactive type (n = 23)							
At rest	1145 ± 31.8	96 ± 3.0	158 ± 7.2	106 ± 8.0	404 ±10.4	432 ±14.8	584 ± 23.3
During CHT	1371 ± 30.9**	113 ± 8.3*	178 ± 9.1**	110 ± 9.1	411 ±19.0	414 ± 15.0	796 ± 25.8**
Areactive type (n = 24)							
At rest	1066 ± 29.5	96 ± 7.0	149 ± 11.0	107 ± 9.1	392 ± 12.6	425 ± 11.0	525 ± 19.4
During CHT	1063 ± 25.4	94 ± 8.3	151 ± 10.0	101 ± 10.5	388 ± 11.0	409 ± 8.4	524 ± 18.1
Paradoxical type (n = 15)							
At rest	1245 ± 37.0	97 ± 7.6	152 ± 6.2	97 ± 6.1	406 ± 8.3	405 ± 7.1	687 ± 18.9
During CHT	1056 ± 22.6**	95 ± 8.7	150 ± 5.5	95 ± 5.8	387 ± 10.2*	420 ± 7.5*	523 ± 13.5**

Note: reliability of differences between the initial state and during CHT. * — $p < 0.05$, ** — $p < 0.01$.

reactivity, which was reflected in a smaller growth of DBP during CHT. On the contrary, after the course, SBP increased significantly in the context of CHT (Table 3), accompanied by an increase in pulse pressure, and, consequently, stroke volume of the left ventricle.

Thus, adaptation to cold-hypoxia effects decreases reactivity of the cardiovascular system somewhat. However, there were participants whose reactivity did not change after the course; the possible reasons behind this are their individual characteristics, including genetic ones [12–14].

Changes in myocardial conductivity caused by swimming in open cold water

We analyzed state of the cardiovascular system after two relay swims. The first swim from Elagin Island to Kronshtadt took place on October 20, 2019, when the water temperature was +7.5–9 °C. The distance was 25 km. Four experienced winter swimmers (aged 37–52 years) participated in the relay swim. As prescribed by the rules of the International Winter Swimming Association (IWSA), the participants swam without wetsuits. Each individual swim lasted 20 minutes, with 60 minutes of rest inbetween.

We recorded ECG 30 minutes before the swim and at the 30th minute of the post-swim recovery. Under the influence of cold and physical activity, two out of four athletes had significantly longer QTc interval, i.e., ventricular conduction slowed down in them (Table 4).

The second relay swim took place at the Oreshek Kronstadt site on June 12–13, 2021. Same 4 swimmers participated therein. They covered 103 km in 22 hours and 16 minutes. The air temperature varied from +16 to 22.3 °C. The water temperature in the Neva river and the Gulf of Finland was +16–17 °C. The swims lasted for 30 minutes, the rest between them — 90 minutes. The analysis of ECG revealed a statistically significant growth of the QTc indicator in 3 out of 4 swimmers (Table 5), while one athlete had it decreasing significantly.

After both swims, no swimmer exhibited significant changes in the BP at the 30th minute of recovery. Thus, in the context of the 2nd swim (t = +17 °C), before it the overall SBP was 119.4 ± 7.3 mmHg, DBP — 78 ± 4.5 mmHg, and post-swim SBP was 123.3 ± 8.5 mmHg, DBP — 73 ± 5.3 mmHg,

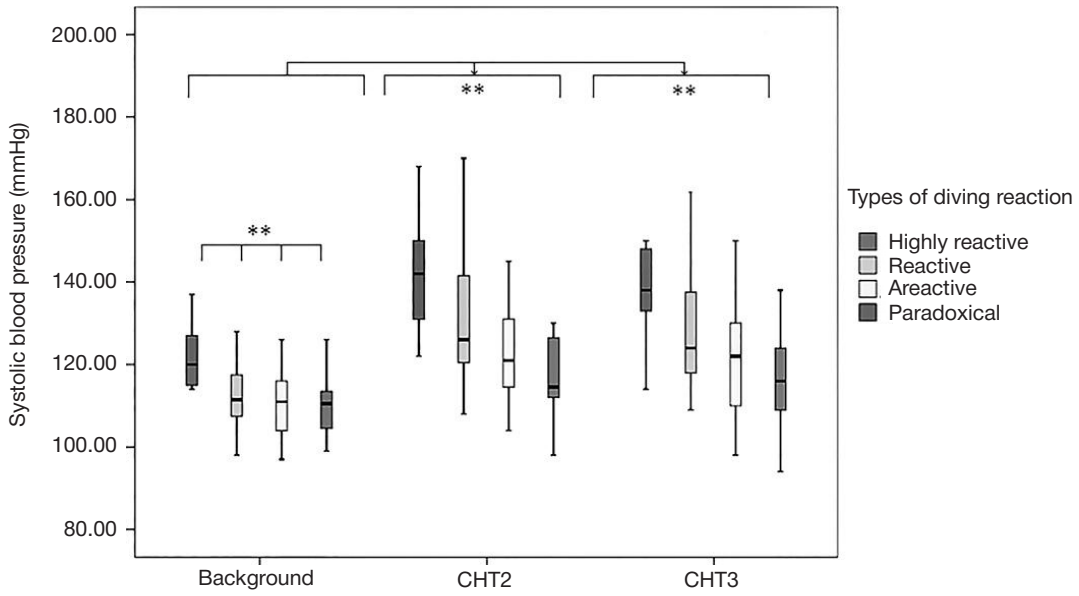

Fig. 4. CHT-triggered changes in systolic blood pressure. Abscissa axis: background — resting state, lying back up; CHT2 — second face immersion test; CHT3 — third face immersion test. Ordinate axis — systolic pressure, mmHg ** — $p < 0.01$ — significance of differences. Highly reactive type — n = 18; reactive — n = 23; areactive — n = 24; paradoxical — n = 15

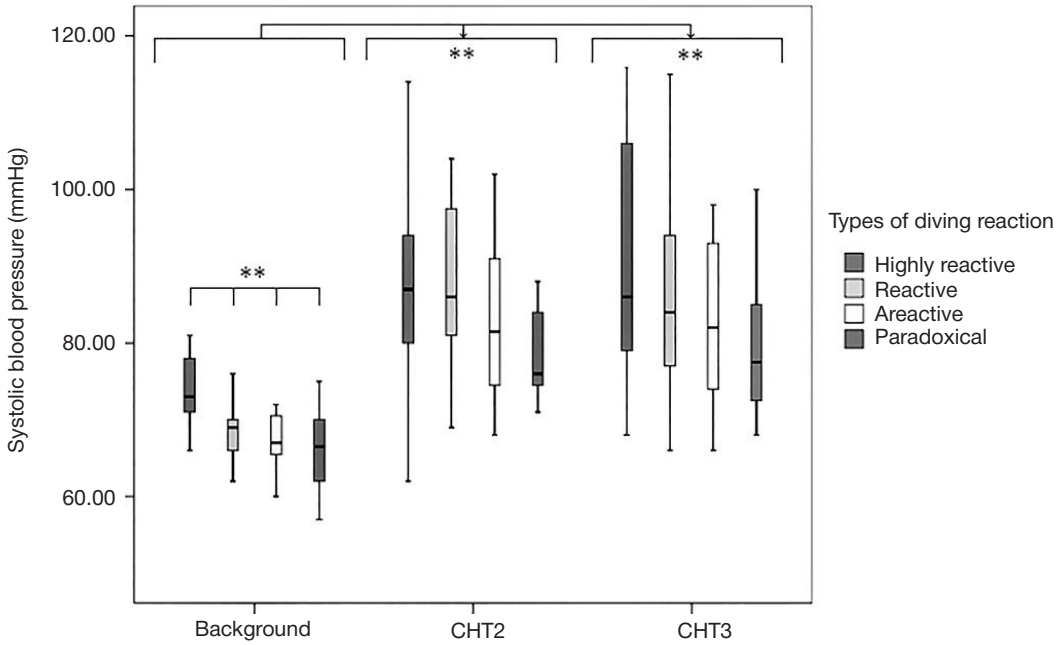

Fig. 5. CHT-associated changes in diastolic blood pressure. Second (CHT2) and third (CHT3) immersions. Ordinate axis — diastolic pressure, mmHg. Other indicators as in Fig. 5. ** — $p < 0.01$. Highly reactive type — $n = 18$; reactive — $n = 23$; areactive — $n = 24$; paradoxical — $n = 15$

respectively. However, control of the athletes' BP pressure dynamics ($n = 17$) short after swimming 200 m (at 3rd–5th minutes of recovery) at water temperature of +1.5-2.5 °C revealed significant changes. Overall, we registered significant growth of SBP in the group. At rest, SBP was 134.4 ± 6.1 mmHg, after swimming — 148.5 ± 5.2 mmHg ($p < 0.05$); DBP at rest = 79.8 ± 3.1 mmHg, after swimming — 91.3 ± 7.1 mmHg ($p < 0.05$). However, in some swimmers, the pressure changed only slightly, but in others, SBP grew up to 190 mmHg, and DBP to 120 mmHg. The data describing these individuals were excluded from the processed pool. After swimming 200 m, heart rate of the athletes increased: at rest it was 76.8 ± 4.4 beats/minute, after swimming — 98.1 ± 4.7 beats/minute ($p < 0.01$). For one participant, cold water swimming translated into heart rate slowdown: before the swim it was 88 beats/minute, after the swim — 64 beats/minute.

DISCUSSION

The analysis of cold-hypoxia tests conducted in the laboratory showed that even when it is only the person's face immersed in water, there occurs a complex of reactions that reflect considerable changes in the cardiovascular system's operation. The reason behind this phenomenon is the diving reflex [17–19], which includes a set of actions simultaneous with activation of the sympathetic and parasympathetic parts of the ANS that send signals to the myocardium. The total effect of stimuli acting on the sinoatrial node from n. vagus and postganglionic sympathetic neurons also depends on the background state of cells of the sinoatrial node, which is shaped by various neuropeptides secreted by vascular endothelium and cardiomyocytes. The magnitude of the cardiovascular system's response to cold water swimming varies depending not only on external factors, such as water temperature, degree of adaptation to cold, etc., but also on the individual characteristics of the body's vegetative reactivity. Diving reflex causes parasympathetic bradycardia, whereas cold stress activates sympathetic tachycardia. These effects, although different in nature, can lead to arrhythmias [7, 8], especially in people with pronounced diving bradycardia. Thus, in some individuals of the highly reactive and reactive types, reflex parasympathetic stimuli affecting sinus node of the heart, in the presence of a pronounced bradycardia, can cause atrioventricular blockade, when impulses cannot reach the ventricles from the atria, which pushes the PQ interval beyond the norm. In some people of the paradoxical type that react to CHT as if it were a stress (expanding sympathetic stimuli to the myocardium), intraventricular conduction deteriorates against the background of a shorter cardiac cycle, i.e., QTc exceeds the norm. This reaction is common for open cold water swims, registered, inter alia, among experienced swimmers adapted to cold water. These data confirm the current understanding that has the diving reflex possessing arrhythmogenic power [6–8]. In addition, these deviations may aggravate against the background of disruption of K^+ metabolism associated with physical exertion during swims, as well as hypothermia while in the water and several minutes afterwards, when the body temperature continues to decrease, including that of the core. These factors may increase the risk of QTc reaching critical values that can lead to cardiac arrest [20, 21].

Table 3. Changes in blood pressure levels (mmHg) caused by the 6-week cold-hypoxia training course ($n = 40$)

Indicators	SBP at rest	DBP at rest	SBP During CHT	DBP During CHT	SBP recovery	DBP recovery
Before training	108.3 ± 4.1	62.7 ± 4.4	122.2 ± 6.7°	84.2 ± 5.3°°	105.8 ± 4.9	60.9 ± 4.1
After training	110.4 ± 7.8	62.1 ± 3.6	135.3 ± 5.1*°°	71.2 ± 4.8*°	104.5 ± 6.1	58.7 ± 4.3

Note: °,* — $p < 0.05$; °° — $p < 0.01$; * — significance of differences before and after the training course; ° — significance of differences between CHT-associated and initial state indicators.

Table 4. Changes in cardiac conduction before and after swimming (water temperature t = +8 °C)

ECG indicators	Swimmer 1		Swimmer 2		Swimmer 3		Swimmer 4	
	Before	After	Before	After	Before	After	Before	After
R-Rcp	696 ± 32	693 ± 37	662 ± 37	584 ± 22*	560 ± 25	648 ± 27*	1088 ± 42	781 ± 25*
P-Q, ms	172 ± 8.1	174 ± 7.1	140 ± 4.2	148 ± 5.2	152 ± 4.4	164 ± 5.1*	158 ± 3.9	165 ± 4.3
QRS	86 ± 4.3	106 ± 5.3*	80 ± 4.9	86 ± 5.1	84 ± 3.6	86 ± 4.2	107 ± 6.1	102 ± 5.9
QTc	457 ± 19	501 ± 21*	447 ± 17	448 ± 22	445 ± 23	498 ± 25*	402 ± 21	420 ± 27

Note: before — initial state before the swim, after — 30–40 minutes of recovery after the relay swim. * — $p < 0.05$, Student's *t*-test used to analyze individual post-swim data against the initial state (ECG recording time — 5 minutes).

Peripheral vessels constrict in response to signals associated with the diving reflex that originate in the vasomotor center of medulla oblongata and move along adrenergic sympathetic fibers to the vessels' muscle walls. Some of the participants, mainly those of the highly reactive type, exhibit growth of BP progressing with each subsequent face immersion in cold water, which is associated with a slow recovery of tone of peripheral vessels that fail to return to their original state within a two-minute interval between dives. We observed a similar reaction in some winter swimmers, when successive swims in cold water with short breaks between them lead to persisting growth of blood pressure. Further examinations in the laboratory setting that involved CHT have confirmed this fact.

Does adaptation to immersion in cold water change the nature of the cardiovascular response? A 6-week training course implying daily sessions of immersion of face in cold water has shown to somewhat improve peripheral vasospasm, which is reflected in a significantly smaller increase of DBP during CHT. On the contrary, SBP increases significantly during CHT, same as pulse pressure, which indirectly reflects expansion of the left ventricle's stroke volume. Against the background of the developing bradycardia, reaction allows maintaining cerebral blood flow at the required level [19, 22–25]. Thus, these changes are adaptive and protective in nature. Reactivity of the heart's chronotropic function CHT also decreases after adaptation. But in some subjects, both cardiac and vascular reactivity remained high, which may be due to their genetic characteristics, in particular — those of the vascular response effector link. Reflex-driven regulation of tone of vessels in the skin is controlled by the sympathetic part of the autonomic regulation with the help of α_1-adrenoreceptors (constrictor function) and β_2-adrenoreceptors (dilator function). The degree of reflex response depends on the ratio of these receptors and the effectiveness of their functioning, which are largely determined on the genetic level. However, there is another factor that affects reflex-driven vascular reactions: biochemical background, which depends, in particular, on the activity of the renin-angiotensin and kinin-bradykinin systems. Thus, using CHT, we have shown that in the context of the diving reflex vascular tone and blood pressure significantly depend on the polymorphism of genes encoding ADRA1A (rs1048101), BDKRB2 (rs1799722), ADBR2 (rs1042713), and ACE (I/D, rs4340) [12, 13].

CONCLUSIONS

Disruption of operation of the cardiovascular system pose the greatest risk associated with cold water swimming. It should be remembered that, under the influence of cold water, practically healthy people with adequate body response to physical exertion may have the functions of their cardiovascular systems changing pathologically, with development of cardiac arrhythmias, including fatal ones, blood pressure spikes and the consequences thereof. Body's regulatory systems, autonomic nervous system in particular, can trigger reflex defense mechanisms in specific ways that increase the risk of a pathological response to a cold stimulus. The key factor is the increased reactivity to extreme stimuli of the autonomous regulatory circuit and its effector link, the myocardium, as well as the smooth muscle walls of peripheral vessels of the skin, non-functioning muscles, and the gastrointestinal tract. In this connection, it is necessary to develop additional tests and criteria for winter swimming athletes' training and competition admission examinations. In our opinion, CHT, carried out with ECG recorded and blood pressure controlled, can be very informative for identifying people facing higher risks on the part of the cardiovascular system.

Table 5. Changes in cardiac conduction before and after swimming (water temperature t = +17 °C)

ECG indicators	Swimmer 1		Swimmer 2		Swimmer 3		Swimmer 4	
	Before	After	Before	After	Before	After	Before	After
R-Rcp	927 ± 25	1105 ± 39**	718 ± 27	674 ± 23	961 ± 29	637 ± 19**	956 ± 31	789 ± 41**
P, mc	120 ± 3.7	114 ± 4.1	124 ± 4.1	116 ± 4.2	124 ± 7.1	110 ± 7.3	114 ± 4.5	110 ± 3.1
P-Q, ms	168 ± 4.3	169 ± 5.1	177 ± 4.1	162 ± 4.7*	173 ± 7.1	160 ± 7.4	150 ± 4.3	146 ± 3.9
QRS	94 ± 5.9	83 ± 4.9	85 ± 3.7	90 ± 4.1	97 ± 4.9	96 ± 4.7	88 ± 4.4	88 ± 4.1
QT, ms	464 ± 17	429 ± 18	371 ± 11	372 ± 17	423 ± 23	394 ± 21	400 ± 17	390 ± 15
QTc	482 ± 28	393 ± 29*	440 ± 7.5	459 ± 11*	434 ± 24	492 ± 25**	405 ± 16	439 ± 15*

Note: before — initial state before the swim, after — 30–40 minutes of recovery after the relay swim. * — $p < 0.05$; ** — $p < 0.01$, Student's t-test used to analyze individual post-swim data against the initial state (ECG recording time — 5 minutes).

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ASSESSMENT OF LIPID SPECTRUM AND C-REACTIVE PROTEIN IN PEOPLE WORKING IN THE ARCTIC ZONE OF RUSSIA

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Adaptation to the extreme living conditions of the North causes dyslipidemia, a risk factor for cardiovascular diseases (CVD), in people working there. This study aimed to assess the level of lipids and C-reactive protein (CRP), a marker of inflammation in CVD cases, in the blood of men staying in the Arctic and Subarctic zones of Russia. Accordingly, the sample was divided into two group, Arctic and Subarctic, the former included 51 participants, aged 35.7 ± 0.6 years, the latter — 54 individuals, aged 34.2 ± 0.9 years (p = 0.167); the duration of their work/stay in the Arctic and Subarctic zones was 7.1 ± 0.2 and 6.4 ± 0.6 years (p = 0.447), respectively. We sampled blood of the participants and measured triglycerides, total cholesterol, low (LDL) and high (HDL) density lipoproteins, atherogenic index (AI), CRP content. Arctic group had higher levels of triglycerides (1.71 ± 0.03 and 1.38 ± 0.14 mmol/l, p = 0.021), total cholesterol (6.15 ± 0.08 and 5.47 ± 0.14 mmol/l, p =0.001), HDL (1.5 ± 0.06 and 1.1 ± 0.04 mmol/l, p = 0.001); the values of LDL did not differ significantly between the groups (4.07 ± 0.08 and 4.1 ± 0.15 mmol/l, p = 0.88), and AI and CRP values (3.41 ± 0.18 and 4.18 ± 0.2, p = 0.007; 3.41 ± 0.18 and 4.91 ± 0.22 mg/l, p = 0.006, respectively) were greater in the Subarctic group. By triglycerides, dyslipidemia was diagnosed in 49.0% and 18.4% of Arctic and Subarctic participants, respectively, by total cholesterol — in 98.0% and 57.8%, by LDL — in 94.1% and 88.0%. As for HDL, their level was lower than normal in 2.0% of the Arctic group subjects and 36.7% of the Subarctic group subjects, which means a higher risk of cardiovascular diseases in the Subarctic region. The level of CRP indicated that 90% of the Arctic group participants were at risk of CVD (moderate risk for 23.5%, high risk for 66.7%), and in the Subarctic group this number was 100% (moderate risk for 7.7%, high risk for 88.5%). The likely reasons behind this are the specifics of nutrition and living conditions. Program of prevention of CVD in the Arctic zone should include lipid profile and CRP tests as part of every periodic medical examination, regardless of age. It is necessary to implement dyslipidemia alimentary correction measures.

Keywords: Arctic zone, lipids, C-reactive protein, cardiovascular risk

Author contribution: Rakhmanov RS — study design and concept, article authoring; Bogomolova ES — editing, approval of the final version of the article; Narutdinov DA — primary material collection; Razgulin SA — literature analysis; Potekhina NN — participation in statistical processing of the material.

Compliance with the ethical standards: the study was approved by the Ethics Committee of the Privolzhsky Research Medical University of the Ministry of Health of the Russian Federation (Minutes #4 of March 14, 2022); all study participants signed a voluntary informed consent form.

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ОЦЕНКА ЛИПИДНОГО СПЕКТРА И С-РЕАКТИВНОГО БЕЛКА КРОВИ У РАБОТАЮЩИХ В АРКТИЧЕСКОЙ ЗОНЕ РОССИИ

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У людей, работающих на Севере, при адаптации к экстремальным условиям жизни развивается дислипидемия, фактор риска при сердечно-сосудистых заболеваниях (ССЗ). Целью работы была оценка уровня липидов и С-реактивного белка (СРБ), маркера воспаления при ССЗ, в крови у мужчин в Арктической зоне России. В крови двух групп: в Арктике (n = 51) и Субарктике (n = 54) (возраст — 35,7 ± 0,6 и 34,2 ± 0,9 лет (p = 0,167), длительность работ — 7,1 ± 0,2 и 6,4 ± 0,6 лет (p = 0,447)) определяли значения триглицеридов, общего холестерина, липопротеидов низкой (ЛПНП) и высокой (ЛПВП) плотности, коэффициента атерогенности (КА), СРБ. В Арктике выявлены более высокие уровни триглицеридов (1,71 ± 0,03 и 1,38 ± 0,14 ммоль/л, p = 0,021), общего холестерина (6,15 ± 0,08 и 5,47 ± 0,14 ммоль/л, p = 0,001), ЛПВП (1,5 ± 0,06 и 1,1 ± 0,04 ммоль/л, p = 0,001); равные значения — ЛПНП (4,07 ± 0,08 и 4,1 ± 0,15 ммоль/л, p = 0,88); менее значимые получены по КА (3,41 ± 0,18 и 4,18 ± 0,2, p = 0,007) и СРБ (3,41 ± 0,18 и 4,91 ± 0,22 мг/л, p = 0,006). Дислипидемия определена по триглицеридам у 49,0% и у 18,4%, по общему холестерину — у 98,0% и 57,8%, по ЛПНП — у 94,1% и 88,0%. ЛПВП ниже нормы у 2,0% и 36,7%, что указывает на более высокий риск сердечно-сосудистых заболеваний в Субарктике. Риск по СРБ в Арктике — у 90% (средний — у 23,5% и высокий — у 66,7%), Субарктике — у 100,0% (средний — у 7,7%, высокий — у 88,5%). Вероятно, это обусловлено особенностями питания и условий жизни. Для профилактики ССЗ в Арктической зоне исследование липидов и СРБ крови необходимо проводить при каждом периодическом медицинском обследовании независимо от возраста. Требуется алиментарная коррекция дислипидемии.

Ключевые слова: Арктическая зона, липиды, С-реактивный белок, сердечно-сосудистый риск

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Dyslipidemia is one of the risk factors for cardiovascular diseases (CVD) [1]. The pathogenesis of CVD includes not only lipid metabolism disorders, but also inflammation, with C-reactive protein (CRP) being one of the most important markers thereof [2, 3]. It can participate in all stages of development of atherosclerotic process [4, 5]. CRP test is part of both primary (distribution into CVD risk groups, qualification for statin therapy) and secondary CVD prevention programs (prognosis of CVD and treatment complications, evaluation treatment efficacy in moderate CVD risk groups) [6].

Extreme cold causes polar hypoxia, which ups body's energy metabolism and switches nutrients processing from carbohydrate to lipid type. Thus, a polar metabolic type is formed [7]. The traditional way of life and nutrition of indigenous people of the North enable adaptation to extreme climatic and geographical factors and prevent cardiovascular and other metabolic diseases. Individuals not native to that zone and arriving there develop specific biochemical changes in the body manifesting as hormonal and metabolic shifts [8, 9].

This study aimed to evaluate the lipid spectrum and the content of C-reactive protein in blood of men working in the Arctic zone of Russia.

METHODS

The study was conducted in July–August 2022 and involved two groups of men working in the Arctic and Subarctic zones, 73rd parallel north (n = 51, group 1) and 69th north latitude (n = 54, group 2), respectively. The inclusion criteria were lack of history of cardiovascular diseases, obesity, inflammatory process in the body. Indigenous people of the North practicing the traditional way of life, as well as people that temporarily left the Arctic zone, were excluded from the study. The participants were practically healthy people aged 35.7 ± 0.6 and 34.2 ± 0.9 years (p = 0.167), undergoing routine periodic examination. None of them expressed any health complaints at the time of examination. Their duration of stay in the Arctic and Subarctic conditions was, respectively, 7.1 ± 0.2 and 6.4 ± 0.6 years (p = 0.447).

We evaluated the conditions of living and work environment of the participants. Almost all of them were smokers. As for the body mass index, no one was obese or underweight. Group 2 worked in the city of Norilsk, which is a zone with anthropogenic pollution [9, 10].

All participants donated blood samples in Norilsk; unfrozen, they were brought to the airport of Norilsk, flown to Krasnoyarsk therefrom, then delivered to the Central Research Laboratory of Krasnoyarsk State Medical University, and analyzed there.

We used an AU5800 analyzer (Beckman Coulter; USA) to establish the lipid metabolism parameters (triglycerides (TG), total cholesterol (TC), low and high density lipoproteins (LDL, HDL), atherogenic index (AI), and a Cobas Integra 400 Plus analyzer (Roche Diagnostics; Switzerland) to reveal the levels of C-reactive protein.

Triglycerides reference values: 1.7 mmol/l; 1.7–2.25 mmol/l — moderately elevated, 2.26–5.65 mmol/l — elevated. TC reference values: 3.5–5.2 mmol/l; 5.2–6.2 mmol/l — borderline high; > 6.2 mmol/l — high. LDL reference values: up to 3.37 mmol/l; 3.37–4.27 mmol/l — elevated; > 4.27 mmol/l — high. HDL reference values: 0.9–1.3 mmol/l [1]. Normal atherogenic index value — ≤ 3.5. CRP reference values — up to 6 mg/l. The levels < 1.0 mg/l, 1.0–2.9 mg/l, ≥ 3.0 mg/l were associated with low, medium and high risk of occurrence and progression of CVD [11, 12]. We established means of the considered indicators that accord with the reference values or are below/above the respective ranges.

The primary data acquired were processed with a Statistica 6.1 software package (StatSoft; USA). We used the Kolmogorov-Smirnov test to check whether the distribution of values is normal or not, established means and standard errors, (M ± m), applied Student's t-test (p < 0.05) to confirm/disprove reliability of differences in the parametric samples, and analyzed individual indicators.

Using the averaged statistical data peculiar to the Arctic, we investigated the relationship between lipidograms and CRP indicators, i.e., established the Pearson correlation coefficients (rxy) and their statistical reliability. With values ranging from 0 to 0.3, the linear connection was considered weak, from 0.3 to 0.5 — light, from 0.5 to 0.7 — moderately strong, from 0.7 to 0.9 — high, and from 0.9 up — very strong.

RESULTS

The conditions of living and work environment were different between the groups. In the Arctic zone, the participants ate in the canteens, their meals were cooked from canned food; additionally, they received foodstuffs as prescribed for people working in the Far North [13]. Drinking water was melted. Accommodation was provided in the specially equipped modules. They worked 24-hour shifts with 48 hour of rest between them, in enclosed spaces as well as in the open (hard, strenuous labor). In the Subarctic zone, the participants lived in comfortable urban apartments, and worked in rooms that meet hygienic standards. Their food was homemade. Fresh vegetables, fruits, berries were consumed rarely; they ate fish twice or thrice a week. However, the food intake pattern was uneven, with 47.3% of group 2 subjects having 3 meals a day, and 52.7% — 2 meals a day; anthropogenic environmental pollutants had an obvious effect on their living. The work of these men was strenuous, but implied little motor activity.

The lipid metabolism data allowed identifying statistically significant differences among a number of means (Table 1): in group 1, the average level of TG was higher by 24.6%, total cholesterol — by 12.4%, HDL — by 36.5%, but AI — 22.6% lower than in group 2.

As for the individual indicators, 51.0% of group 1 participants had TG within the normal range, same as 81.6% of men from group 2. Accordingly, TG tests returned moderately elevated values for 47.1% and 4.1% of the blood samples, and elevated for 2.0% (1 person) in the Arctic group and 14.3% in the Subarctic group (Table 2). In the latter group, the proportion of samples with moderately elevated TG level was more significant: higher by 8.3%.

The level of total cholesterol in the Arctic group was normal in 1 person only, while in the Subarctic group it was within the normal range in 42.2% of the participants. Borderline TC values were registered in 60.8% and 24.4% of subjects, high values — in 37.3% and 33.3%, respectively (Table 3). Overall, group 2 had less borderline TC occurrences and similar number of high TC cases.

In group 1, the level of LDL was normal in 5.9% of the participants, in group 2 — in 12.0%; it was elevated in 64.7% and 48.0% of them, and high — in 29.4% and 40.0% of the subjects, respectively (Table 4). We registered no LDL values beyond the reference range in either of the groups.

In group 1, 35.3% of the participants had HDL within the normal range, 62.7 — above normal, less than 2.0% — below normal. In group 2, the respective figures were 48.3% (normal), 32.3% (above normal), 19.4% (below normal).

As for the AI, it was normal in 56.8% of group 1 participants and 29.4% of group 2 subjects, while 43.1% and 70.6%, respectively, had it above the normal range (Table 5). There was significant difference between normal and high AI values.

Table 1. Lipid metabolism indicators, both groups, absolute values

№	Lipid spectrum indicators	Arctic zone, M ± *m*		*p*
		Arctic	Subarctic	
1	Triglycerides	1.72 ± 0.03	1.38 ± 0.14	0.021
2	Total cholesterol	6.15 ± 0.08	5.47 ± 0.14	0.001
3	Low-density lipoproteins	4.07 ± 0.08	4.1 ± 0.15	0.88
4	High-density lipoproteins	1.5 ± 0.06	1.1 ± 0.04	0.001
5	Atherogenic index	3.41 ± 0.18	4.18 ± 0.2	0.007

Table 2. Blood plasma triglycerides, both groups, absolute values

№	Arctic Zone	Assessment, M ± *m*		
		Normal	Moderately elevated	Elevated
1	Arctic	1.61 ± 0.03	1.8 ± 0.01	2.56
2	Subarctic	1.0 ± 0.07	1.95 ± 0.07	3.31 ± 0.4
p		0.001	0.001	–

In group 1, only 9.8% of the participants had low CRP, while in 23.5% the level thereof was moderate, and in 66.7% — high. In group 2, these values were 0%, 7.7% and 88.5%, respectively (Table 6).

Searching for correlations between lipid metabolism and CRP, we established only one, with TG, which was moderately strong, negative, statistically significant (Table 7).

The correlations between individual indicators of the lipid spectrum and the AI turned out to be interesting. We established a significant positive strong relationship between TC, LDL and TG, but in case of HDL, it was insignificant and weak. Triglycerides had a moderately strong significant positive association with LDL. We have also identified significant relationships between AI and HDL (negative, rather strong), AI and LDL (moderately strong, positive) (Table 8).

DISCUSSION

The land and marine areas comprising the Arctic zone of Russia are stipulated by the Russian legislation [14]. Extreme weather and climatic conditions that undermine health and influence morbidity, mortality, and working capacity of the population are common to all of those areas [15–21].

People coming to the North and staying there for a long time begin to adapt to the said conditions; rearrangement of lipid metabolism is one of the aspects of that adaptation, and it entails dyslipidemia. Several studies report that already in the first year of living in the high latitudes, TC grows up to borderline-high and high values. This is when the body responds with mobilization of its reserves, which manifests in the increasing level of HDL and prevents atherogenic changes. However, after five years in the North, residents not native to this zone start suffering dyslipidemia with hyperglyceridemia, high TC and LDL levels, while those of HDL in them decrease 1.4-fold compared to the first year [22, 23].

The participants of our study, who came to the Arctic zone to work there, had the mean level of HDL high for a longer period of time than registered in non-native residents, which indicates an adequate mobilization of the body's reserves in response to the conditions of the North. In group 1, the proportion of people with such a level of HDL was 1.9 times higher, and with a low level — 9.7 times less, which shows that those working in the Arctic adapt better. Comparing the Arctic and Subarctic groups, we may conclude that higher share of those with elevated HDL levels in the former points to a greater importance of the compensatory action of lipid metabolism in that group, as confirmed by the AI.

Dyslipidemia is associated with an increased risk of cardiovascular events [24]. There is evidence confirming the inverse relationship between HDL levels and the risk of coronary heart disease [25]. HDL condition reverse cholesterol transport from arterial wall and peripheral tissues to the liver, protect LDL from oxidation, produce anti-inflammatory and vasodilating effects on vascular wall cells [26]. Thus, a significant proportion of those working in the Subarctic had the HDL perform their protective functions to a lesser degree. The list of reasons for low plasma HDL includes insufficient intake of cholesterol with food and low motor activity [27]. Anthropogenic environmental pollutants may have caused the decrease of levels of HDL, but it is impossible to arrive at such a conclusion without further research.

Table 3. Plasma cholesterol, both groups, absolute values

№	Arctic Zone	Assessment, M ± *m*		
		Normal	Borderline	High
1	Arctic	5.11	5.83 ± 0.04	6.74 ± 0.11
2	Subarctic	4.41 ± 0.15	5.49 ± 0.09	6.64 ± 0.1
p		–	0.011	0.542

Table 4. Low-density lipoproteins, both groups, absolute values

№	Arctic Zone	Assessment, M ± *m*		
		Normal	Borderline	High
1	Arctic	2.8–3.3	3.83 ± 0.04	4.77 ± 0.1
2	Subarctic	1.79–2.36	3.73 ± 0.05	4.86 ± 0.08
p			0.122	0.498

Table 5. Atherogenic index, both groups

№	Arctic Zone	Assessment, M ± *m*	
		Normal	Above normal
1	Arctic	2.56 ± 0.13	4.47 ± 0.2
2	Subarctic	2.68 ± 0.1	4.46 ± 0.16
p		0.729	0.988

Table 6. C-reactive protein levels, both groups, absolute values

№	Assessment	Arctic zone, M ± *m*		*p*
		Arctic	Subarctic	
1	CRP by group	3.41 ± 0.18	4.91 ± 0.22	0.006
2	Low CRP	0.87 ± 0.09 (5)	0.86	–
3	Moderate CRP	2.0 ± 0.17 (12)	2.45–2.89	–
4	High CRP	4.97 ± 0.15 (34)	5.12 ± 0.12	0.467

The pathogenesis of most CVD of athero- and thrombogenic origin involves both lipid metabolism disorders and inflammatory processes; CRP is the leading mediator of the acute phase and a marker of inflammation [2, 28–30]. It is considered a real risk factor for cardiovascular diseases, like TC and LDL, which expands the concept of residual risk of cardiovascular inflammation [30]. CRP deposits in atherosclerotic plaques and damaged tissues [3, 26, 27]. The higher the content of CRP, the greater the association with the relative risk of occurrence and progression of cardiovascular events [11–12]. In our study, CRP values were within the reference range. However, 90% of those working in the Arctic zone ran the risk of CVD, more than 2/3 of them — high risk thereof; for the Subarctic zone, this figure was 100%, with the proportion of those at high risk of CVD 21.8% higher than in the Arctic zone.

The investigation of correlations revealed that CRP has relationship only with TG, which confirms it is an independent CVD risk factor for people working in the Arctic zone.

Thus, the risk of cardiovascular diseases is more pronounced among those working in the Subarctic than in the Arctic zone. The likely reasons behind this are the specifics of nutrition and living conditions. Thus, dyslipidemia requires alimentary correction measures, and, possibly, therapeutic interventions to reduce the level of CRP.

It is recommended to test for CVD risk factors, including dyslipidemia, men aged 40 and above, and women once they turn 50 or begin menopausal transition [1]. Our study highlights the need for lipid spectrum assessments and CRP tests in connection not with age, but with employment in the Arctic zone; such assessments and tests should be done annually, during periodic routine medical examinations, since they would allow timely correction of atherosclerotic and inflammatory changes in the body and reduction of the risk of CVD.

CONCLUSIONS

In the Arctic zone, as compared to the Subarctic, we established higher values of triglycerides (1.71 ± 0.03 and 1.38 ± 0.14 mmol/l, $p = 0.021$), total cholesterol (6.15 ± 0.08 and 5.47 ± 0.14 mmol/l, $p = 0.001$), high-density lipoproteins (1.5 ± 0.06 and 1.1 ± 0.04 mmol/l, $p = 0.001$); equal values of low-density lipoproteins (4.07 ± 0.08 and 4.1 ± 0.15 mmol/l, $p = 0.88$); less significant differences in the atherogenic index (3.41 ± 0.18 and 4.18 ± 0.2, $p = 0.007$) and C-reactive protein levels (3.41 ± 0.18 and 4.91 ± 0.22 mg/l, $p = 0.006$). By triglycerides, dyslipidemia was diagnosed in 49.0% of the Arctic group participants and 18.4% of the Subarctic subjects; by total cholesterol — in 98.1% and 57.7%, by low-density lipoproteins — in 94.1% and 88.0%, respectively. As for HDL, their level was lower than normal in 2.0% and 19.4% the participants, respectively, which points to a higher risk of cardiovascular diseases in the Subarctic region. As shown by the level of CRP, 90% of the Arctic group participants were at risk of CVD (moderate risk for 23.5%, high risk for 66.7%), and in the Subarctic group this number was 100% (moderate risk for 7.7%, high risk for 88.5%). Prevention of cardiovascular diseases and sound basis for decisions related to medical assistance, as they concern people working in the Arctic zone, require lipid spectrum assessments and CRP tests to be part of every periodic routine medical examination, regardless of age.

Table 7. Correlations between lipid spectrum indicators and CRP

№	Lipid spectrum – CRP indicators	Pearson's test	*p*
1	Total cholesterol	–0.022	0.917
2	High-density lipoproteins	–0.06	0.675
3	Low-density lipoproteins	–0.081	0.588
4	Triglycerides	–0.453	0.02
5	Atherogenic index	0.097	0.497

Table 8. Correlations between lipid spectrum indicators

№	Indicator	HDL		LDL		Triglycerides		AI	
		Pearson's test	*p*	Pearson's test	*p*	Pearson's test	*p*	Pearson's test	*p*
1	Total cholesterol	0.282	0.172	0.837	0.001	0.894	0.001	0.164	0.435
2	Triglycerides	0.129	0.528	0.51	0.008	–	–	0.009	0.986
3	Low-density lipoproteins	–0.155	0.298	–	–	0.51	0.008	0.412	0.004
4	Atherogenic index	–0.912	0.001	0.412	0.004	0.009	0.966	–	–

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ASSESSING BIODISTRIBUTION OF BIOMEDICAL CELLULAR PRODUCT BASED ON HUMAN CHONDROCYTES FOLLOWING IMPLANTATION TO BALB/C NUDE MICE

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Despite the prospects of the approach to cell therapy of cartilage damage in humans involving autologous chondrocytes, similar technologies are just beginning to be introduced into medical practice in the Russian Federation. In this regard, the development of biomedical cell products (BCPs) for cartilage tissue repair is quite topical, while the use of organoid technology is the most close to the native tissue conditions. According to requirements of legislation of the Russian Federation, it is necessary to assess biodistribution characterizing migration potential of the cells, their tropism for body tissues following implantation within the framework of preclinical trials. The study was aimed to assess biodistribution of novel BCP based on human chondrocytes in the form of chondrospheres after subcutaneous implantation in Balb/c nude mice. Implantation to 12 mice was performed during the first phase, along with administration of saline to 12 control animals. Weighting and follow-up were conducted for 90 days. Then mice were withdrawn from the experiment to collect samples of organs and tissues for histological analysis of the implant, estimation of its viability, integration. During the second phase biodistribution was assessed by PCR in order to detect human DNA in the organ and tissue samples. Chondrospheres successfully integrated in the tissues surrounding the inoculation zones and formed cartilage tissue. No significant ($p < 0.05$) changes in weight were reported. No human DNA found in chondrosphere implantation zones was detected in the samples collected from other organs and tissues. BCP demonstrated no biodistribution across other tissues and organs of mice 90 days after implantation, which suggested that the product developed was safe.

Keywords: biomedical cellular product, chondrocytes, biodistribution, preclinical trials, chondrospheres

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ИССЛЕДОВАНИЕ БИОРАСПРЕДЕЛЕНИЯ БИОМЕДИЦИНСКОГО КЛЕТОЧНОГО ПРОДУКТА НА ОСНОВЕ ХОНДРОЦИТОВ ЧЕЛОВЕКА ПРИ ИМПЛАНТАЦИИ МЫШАМ ЛИНИИ BALB/C NUDE

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Несмотря на перспективность подхода клеточной терапии повреждений хряща человека с помощью аутологичных хондроцитов, подобные технологии только начинают внедрять в медицинскую практику в Российской Федерации. В связи с этим разработка биомедицинских клеточных продуктов (БМКП) для восстановления хрящевой ткани достаточно актуальна, а использование органоидных технологий наиболее приближено к условиям нативной ткани. Согласно требованиям законодательства РФ, в рамках доклинических исследований необходимо изучение биораспределения, характеризующего миграционный потенциал клеток, их тропность к тканям организма при имплантации. Целью работы было исследовать биораспределение нового БМКП на основе хондроцитов человека в виде хондросфер после подкожной имплантации мышам линии Balb/c nude. На первом этапе осуществляли имплантацию 12 мышам, а также введение физиологического раствора 12 контрольным животным. В течение 90 дней проводили взвешивание и наблюдение, а затем выводили мышей из эксперимента для получения образцов органов и тканей для гистологического анализа импланта, оценки его состоятельности, интеграции. На втором этапе изучали биораспределение методом ПЦР для выявления ДНК человека в образцах тканей и органов. Хондросферы успешно интегрировались в окружающие ткани зоны инокуляции, формировали хрящевую ткань. Статистически значимых ($p < 0,05$) изменений в весе не зафиксировали. В образцах из зоны имплантации хондросфер была выявлена ДНК человека, которую не обнаруживали в других органах и тканях. БМКП через 90 дней после имплантации демонстрировал отсутствие биораспределения в другие ткани и органы мышей, что свидетельствует о безопасности разрабатываемого продукта.

Ключевые слова: биомедицинский клеточный продукт, хондроциты, биораспределение, доклинические исследования, хондросферы

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Advances of recent years in the development of various approaches to cell therapy for cartilage tissue damage enable treatment of some joint disorders, including disorders associated with human cartilage lesions of multifactorial etiology [1]. Implantation of autologous chondrocytes is considered to be an effective and promising method to treat cartilage tissue damage with minimal risk of adverse events [2]. The principle of this procedure consists in scaling up and culturing chondrocytes obtained from the biopsy specimen of the patient's articular cartilage fragment with subsequent transplantation of cells immediately into the defect [2]. The researchers became more and more interested in cell therapy since the first report of implantation of autologous chondrocytes into the damaged area of human articular cartilage [3]; new methods to modify and optimize this approach appeared over the decade [1]. The use of biomedical cell products (BCPs) based on autologous chondrocytes was superior to more conventional methods, arthroscopic debridement and microdamage, in terms of efficacy [4–7]. Today, a number of BCPs are through various phases of pre-clinical and clinical trials [1, 2, 6, 8, 9]; some products are approved for treatment of the human cartilage focal lesions [11–14].

Spheroids, the 3D structures resulting from self-aggregation of cells cultured under certain conditions, have many advantages over BCPs of other types when used for treatment of articular cartilage defects [15]. Thus, 3D conditions have a beneficial effect on proliferative activity and phenotypic stability of mature chondrocytes [9]. In contrast to suspension of autologous chondrocytes, which become capable of producing extracellular matrix (ECM) only after a certain time after transplantation, the cells comprised in spheroids can secrete the ECM components even in the phase of culturing [16, 17]. In addition, autologous spheroids do not require using third-party biomaterials, they easily integrate into the tissue in the damaged area; there is no need for systemic immunosuppression after implantation [15]. In combination, specified properties of spheroid BCPs can contribute to high-quality filling of the cartilage tissue defect. This, the efficacy of spheroids based on autologous chondrocytes has been demonstrated in animal models [17] and clinical trials [18, 19]. Spheroid BCPs have shown significantly better therapeutic effect in terms of structural cartilage defect restoration compared to the microdamage procedure [6, 7].

Spheroxтм (Co.don; Germany), one of the globally approved cell therapy drugs for restoration of human articular cartilage defects, represents a spheroid BCP. Today, the Generium company produces this drug under license from Co.don and completes phase III clinical trial in the Russian Federation [10]. At the same time, no analogues of such BCPs are available in the Russian Federation Thus, the only product that is, in fact, a technology transfer from Co.don, is currently undergoing clinical trials in the Russian Federation Given optimistic results of the cell therapy trials conducted by foreign partners, the development and introduction of such technology into research and clinical practice in the Russian Federation eems to be very topical.

The main task of working with BCPs is to maintain efficacy and meet the critical quality parameters to ensure safety and the expected effect, which should be predicted before the start of the clinical trial. That is why BCPs, as all other medications, must meet strict requirements to be approved by the authorities for further research and implementation [8]. This requires the development and implementation of appropriate assessment methods to evaluate the cell-based product before and after implantation within the framework of pre-clinical trial [19, 20]. Thus, it is necessary to identify the major risk factors, such as tumorgenicity, carcinogenicity and biodistribution, before the beginning of pre-clinical trial involving animals [21].

Biodistribution is one of the most important safety criteria characterizing migration potential of the cells comprised in BCP after implantation, along with the capabilities of forming ectopic tissue and persisting inside/outside the administration site [9, 22–24]. Biodistribution is usually assessed in immunodeficient animal models; subcutaneous implantation is preferred due to its less invasive nature [24]. Since the tested product should be as close as possible or similar to the final BCP variant based on its properties, it is advisable to avoid the use of fluorescent tags or any other approaches potentially changing the product structure and properties when assessing biodistribution [8].

The study was aimed to assess biodistribution of the Chondrosphere spheroid BCPs designed for treatment of articular cartilage lesions in humans after subcutaneous implantation to the Balb/c Nude immunodeficient mice.

METHODS

Legal regulation

The study represents a preclinical trial of novel BCP conducted in accordance with the current regulatory requirements [21, 25–27]. The study was carried out according to the approved written plan and Standard Operating Procedures. The employees, who took part in the experiment, were trained to ensure proper, humane care and use of laboratory animals.

Spheroid BCPs

The stidued BCPs represented a 3D culture of spheroids based on human chondrocytes obtained using the Aggre Well 800 microwell plate (STEMCELL Technologies; Canada) in accordance with the manufacturer's protocol. The number of cells per microwell of the plate was 4–5 × 10^3. The spheroids obtained (the cellular or tissue-engineered product is referred to as Chondrosphere) were cultured in miniature bioreactors on the 3D orbital shaker (Infors HT; Switzerland) at 37 °C and 5% CO_2 [28]. Advanced DMEM (Gibco, Thermo Fisher Scientific; USA) supplemented with 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 10 ng/ml bFGF (STEMCELL Technologies; Canada), 100X Glutamax (Gibco, Thermo Fisher Scientific; USA), 50X B27 (GIBCO, Thermo Fisher Scientific), 1% Insulin-Transferrin-Selenium (ITS) (PANECO; Russia), 50 μg/ml of ascorbic acid (Sigma Aldrich; USA), 5 μg/ml of plasmocin, gentamicin (PANECO; Russia) and 10 mL/L 100x solution of penicillin/streptomycin (PanEco; Russia) were used as culture medium. Spheroids were cultured for 28 days; the medium was changed every 4 days.

Experimental design

Inbred Balb/c Nude immunodeficient mice were selected for safety assessment. BCPs were administered to animals (n = 12; 6 females, 6 males) by a single subcutaneous injection in the head in a dose of five spheroids in saline (groups 1, 2). In addition, 12 mice (6 males and 6 females) were used as control animals thet received subcutaneous injection of 50 μL of saline (groups 3, 4). The animals were weighted regularly, and the inoculum size was measured in the implantation area during the experiment. Then, 90 calendar days after administration 12 females and 12 males were euthanized by decapitation under inhalation anesthesia. After that specimens from the following organs and tissues were harvested: lymph nodes, thyroid,

Table 1. Primers used in the study

Name	Sequence 5'→3'	Product size
mActb-F	GAT GCA CAG TAG GTC TAA GTG GAG	121
mActb-R	CAC TCA GGG CAG GTG AAA CT	
CO1-F	CAA CCT CAA CAC CAC CTT C	269
CO1-R	CTC GTG TGT CTA CGT CTA TTC	

aorta, heart, lungs, thymus, esophagus, stomach, pancreas, small intestine, large intestine, liver, spleen, kidney, bladder, adrenal glands, brain, testes, ovary, administration site, blood, tumor.

The euthanized animal was treated with 96% ethanol. All subsequent phases of organ harvesting were accomplished under a laminar flow hood in aseptic environment.

Histological analysis

Biomaterial was fixed in the Histosafe 10% formaldehyde solution (BioVitrum; Russia) for 24 h, then washed with running water for 20 min to remove excess fixing agent and dehydrated five times with the Blic modified isopropyl alcohol (BlicMedicalProduction; Russia). Then the specimens were embedded in paraffin. The 4–5 μm histological sections were obtained using the Microm HM325 microtome (Microm; Germany). Paraffin removal was performed in accordance with the following scheme: xylene № 1 — 2 min, xylene № 2 — 2 min, 96% ethanol № 1 — 2 min, 96% ethanol № 2 — 2 min, 70% ethanol — 2 min, distilled water — 2 min. Histological sections were stained with hematoxylin and eosin (Mayer's haematoxylin, eosin 1% aqueous solution (BioVitrum; Russia)). The resulting slices were assessed using the Levenhuk 625 microscope (Levenhuk; Russia).

Genomic DNA isolation

The M-SORB-OOM kit (Sintol; Russia) was used in accordance with the manufacturer's instructions to extract genomic DNA from the organs of mice and human capillary blood to be used as positive control for human DNA. The 10–20 mg fragments of organs or 10–20 μL of capillary blood were used for extraction. Samples with no organ or tissue specimens were used as negative controls. Genomic DNA was eluted in 400 μL of elution buffer. The finite volume of the solution with isolated genomic DNA was 400 μL.

Polymerase chain reaction (PCR)

PCR was performed in the CFX96 Touch system for nucleic acid amplification (Bio-Rad; USA) using the ready-made 5X Screen Mix for PCR (Evrogen; Russia) in accordance with the manufacturer's instructions. We used primers specific for the cytochrome C oxidase subunit I (CO1) genes to detect human DNA and β-actin specific for mice (mActb) to detect murine DNA when performing the reaction (Table 1).

Amplification was performed in accordance with the following protocol:

1) 95 °C — 5 min;
2) 95 °C — 15 s;
3) 58 °C — 15 s;
4) 72 °C — 30 s.

Steps 2, 3 and 4 were repeated in 40 cycles.

Agarose gel electrophoresis

DNA electrophoresis was conducted in 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer in the horizontal electrophoresis chamber (Biorad; USA). Visual detection of amplification products involved the use of 0.5 μg/mL ethidium bromide. Voltage was set as 120 V, and the run time was 20 min. The amplification products were detected with the UV transilluminator (Vilber; Germany).

Statistical analysis

The results of weight estimation in the animal subjects were processed using the Microsoft Excel (Microsoft; USA) and SPSS Statistics 17.0 (IBM; USA) software packages. The Shapiro–Wilk test was used to test the trait distribution for normality. Mann–Whitney U-test was used for comparison. Bonferroni correction for multiple comparisons was applied. The differences between groups were considered significant at $p < 0.05$. Graphs were plotted with the GraphPad Prism software (Dotmatics; USA).

Handling the remaining BCPs

BCPs not used in the experiment were autoclaved and disposed as class B waste.

RESULTS

Morphometric analysis

Regular weighting for 90 days revealed no significant differences in body weight between the groups receiving BCPs and control groups (Fig. 1).

There were no significant differences in the animals' general health between the experimental and control groups. The animals remained active and showed normal feeding behavior.

Histological analysis

After histological staining of speciments from the BCP implantation area we observed stable cartilage tissue with the large number of chondrocytes and the emerging lacunae (Fig. 2). Cell migration from the implantation area was minimal.

Detection of human DNA in murine tissues and organs

The analysis of whole blood samples, mirine organ and tissue specimens revealed human DNA in the chondrosphere injection area only (Table 2). No traces of the tested BCP were detected in other tissues and organs of male and female mice (LOQ < 0.001 ng of DNA). Thus, the BCP biodistribution pattern was optimal for the recommended administration route.

DISCUSSION

Obtaining 3D spheroid BCPs based on autologous human chondrocytes using the organoid technique is considered to be a rather promising direction of the development of products for cell therapy of large focal hyaline cartilage defects [28]. Despite the fact that the composition of the product we are

developing now is similar to that of the product by Generium, it is obtained using a modified technique, which requires safety testing. According to the current standards, the study of BCP pharmacokinetics includes assessment of biodistribution characterizing migration potential of the cells comprised in the construct [27]. Previously, the Spheroxтм product researchers assessed biodistribution of their invention implanted in immunodeficient animals as part of registration activities after consulting with the regulator [8]. The analysis by PCR showed that there were no human DNA in the tissues and organs distant from the subcutaneous implantation site. Thus, it seems reasonable to assess biodistribution of BCPs designed for impantation in humans using the discussed approach within the framework of safety testing.

Our study was aimed to assess biodistribution of the spheroid BCP designed for treatment of human articular cartilage lesions in immunodeficient mice. As far as we know, this is the first large-scale preclinical trial of BCP based on autologous chondrocytes in the Russian Federation.

Balb/c Nude mice were used to assess biodistribution. These immunodeficient mice are widely used in the trials of xenografts, including that based on human chondrocytes [29–30]. We used subcutaneous implantation of spheroids, since this procedure is less invasive, expandable and easier to implement — for example, compared to implantation in the small rodent's joint. A single BCP dose was calculated based on the estimated therapeutic dose for humans in accordance to the cartilage tissue defect size: 10–70 spheroids per 1 cm^2 of damaged tissue [8]. In mice, the dose was five spheroids per animal.

To assess stable cartilage tissue formation in the BCP administration site, the injection sites were examined using histological analysis. We observed cartilage tissue development 90 days after implantation, which was indicative of successful integration of spheroids into murine tissues. Morphometry revealed no significant changes in body weight in the experimental groups, which suggested no systemic morbid effect. Furthermore, there was no abnormal tissue growth associated with carcinogenesis (month 3 of follow-up) or tumorgenesis.

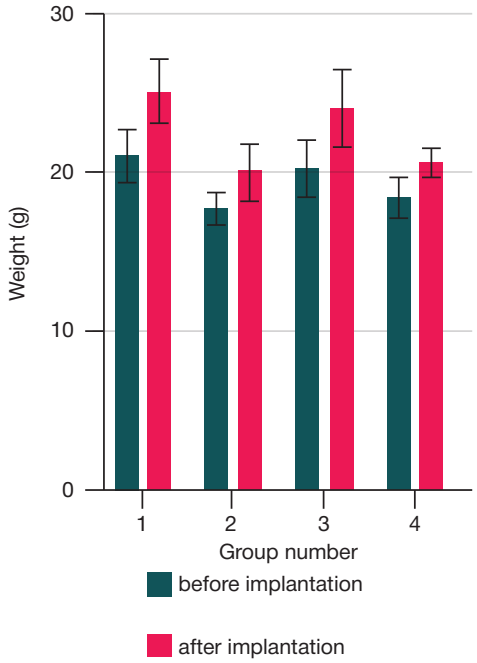

Fig. 1. Changes in the experimental animals' body weight by groups before and after the experiment. 1, 2 — groups of males and females, which underwent BCP implantation, n = 12; 3, 4 — groups of males and females, which received subcutaneous injection of saline, n = 12. * — significant intragroup differences, $p < 0.05$

To assess biodistibution, biopsy specimens of murine organs and tissues were qualitatively tested for expression of the human-specific sequence of the gene encoding cytochrome C oxidase subunit 1 (COI) 90 days after implantation. Our findings showed that a single subcutaneous administration of BCP to experimental mice resulted in the fact that human DNA was detected exclusively in the administration site, not in the other assessed tissues and organs. Thus, human DNA is related exclusively to the cells comprised in the spheroids implanted. However, in the future we plan to assess BCP biodistribution and carcinogenicity in mice throughout a longer period after implantation in order to evaluate potential delayed effects.

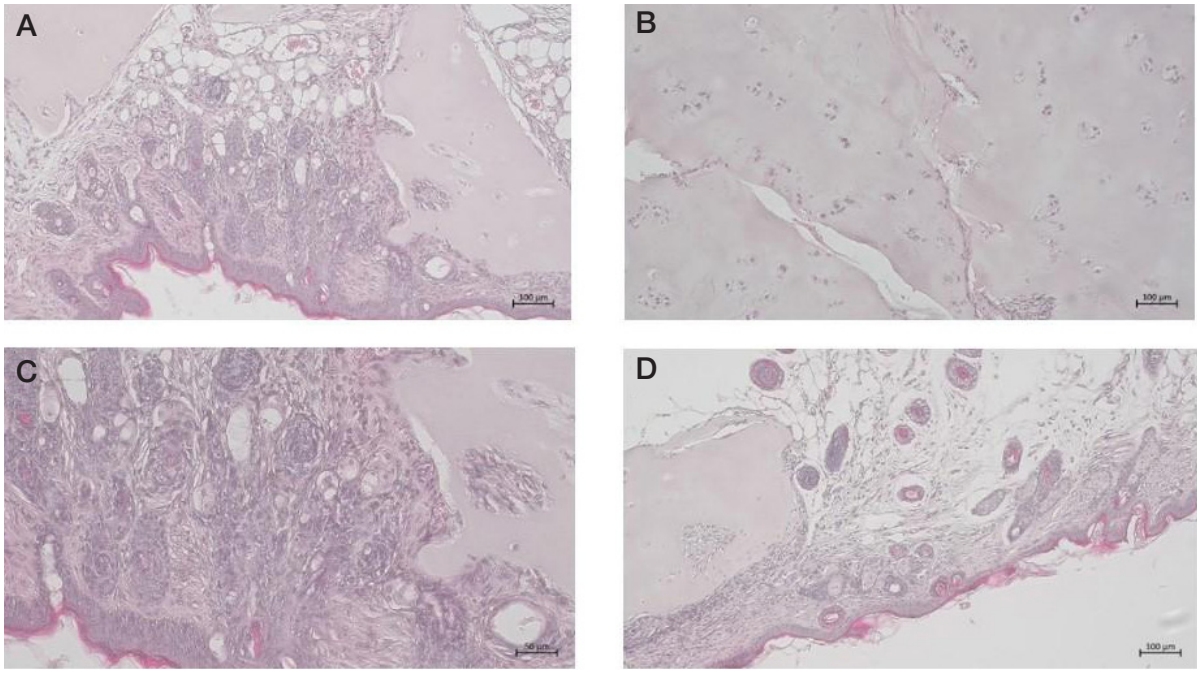

Fig. 2. Chondropheres in murine tissues 90 days after implantation. Hematoxylin and eosin stain. 10x magnification. Scale bar — 100 μm

Table 2. Human DNA detection

Organ/Tissue	Group number (n = 24)			
	1	2	3	4
Lymph nodes	------	------	------	------
Thyroid	------	------	------	------
Aorta	------	------	------	------
Heart	------	------	------	------
Lung	------	------	------	------
Thymus	------	------	------	------
Esophagus	------	------	------	------
Stomach	------	------	------	------
Pancreas	------	------	------	------
Small intestine	------	------	------	------
Liver	------	------	------	------
Spleen	------	------	------	------
Kidney	------	------	------	------
Bladder	------	------	------	------
Adrenal glands	------	------	------	------
Brain	------	------	------	------
Testes	------	------	------	------
Ovary	------	------	------	------
Implantation site	++++++	++++++	------	------
Blood	------	------	------	------
Tumor	------	------	------	------

Note: Positive (indicative of the presence of human DNA) qualitative data were obtained for the samples designated as "+"; 1, 2 — groups that received BCP; 3, 4 — groups that received saline.

The results obtained in this phase suggest no cell migration processes, which indicates that the product developed is safe in terms of biodistribution.

CONCLUSIONS

In this study we assessed biodistribution of BCPs in the form of chondrospheres based on human chondrocytes by subcutaneous implantation to Balb/c Nude mice. During the study we observed the development of stable mature cartilage tissue showing no signs of abnormal proliferation or cell migration outside the implantation site. Such findings allow us to conclude that the BCP developed is characterized by normal biodistribution within the administration site and successful integration into the surrounding tissies. Thus, this cell engineering product, Chondrosphere, can be recommended for further testing.

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COMPARATIVE ANALYSIS OF EFFICACY OF THE NEW LOCAL HEMOSTATIC AGENTS

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Various local hemostatics (based on collagen, gelatin, cellulose, etc.) are used to stop bleeding from parenchymal organs of the abdominal cavity. In the context of an acute *in vivo* experiment, this study aimed to comparatively assess the time and volume of bleeding from a trauma of abdominal cavity's parenchymal organs covered with a new collagen-based spongy hemostatics combined with Na-CMC. We used new multicomponent polymer sponge implants (MPSI) based on marine collagen and carboxymethyl cellulose sodium salt, Na-CMC; the components were mixed in the ratios of 15/85, 25/75, 50/50. Hemostatic activity of the samples was assessed by bleeding time and blood loss volume. For the experiments, rats underwent laparotomy and resection of the left lobe of liver (series 1) and lower pole of spleen (series 2). In both series of experiments, the controlled parameters (bleeding time and blood loss volume) were smallest in group 6, where the MPSI were 50/50 Na-CMC/collagen. The hypothesis of higher efficacy of composite local hemostatic agents (namely, made of Na-CMC and deep-sea squid collagen) in cases of trauma of the parenchymal organs was confirmed experimentally, and same experiment has also shown that collagen in the composition of MPSI boosts bleeding arrest (for liver injury, the smallest blood loss and hemorrhage control time was 41 s, for spleen injury — 57 s, respectively; $p \leq 0.05$).

Keywords: hemostasis, hemostatic sponges, polymers, *in vitro* experiment, bleeding, collagen

Author contributions: Lipatov VA — concept and design, article authoring, editing, approval of its final version; Lazarenko SV — experimental part, statistical processing, article editing, approval of its final version; Severinov DA — experimental part, article editing, approval of its final version; Denisov AA — experimental part, article authoring, literature data analysis; Chupakhin EG, Aniskina EN — experimental part, article authoring, literature analysis.

Compliance with ethical standards: the study was approved by the Ethics Committee (Minutes #3 of November 16, 2020), conducted in compliance with international and national standards for care and use of laboratory animals.

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ ЭФФЕКТИВНОСТИ НОВЫХ ОБРАЗЦОВ МЕСТНЫХ ГЕМОСТАТИЧЕСКИХ СРЕДСТВ

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Для остановки кровотечения из паренхиматозных органов брюшной полости применяют различные варианты местных гемостатических средств (на основе коллагена, желатина, целлюлозы и пр.). Целью работы было провести сравнительную оценку времени и объема кровотечения после травмы паренхиматозных органов брюшной полости с использованием новых образцов губчатых кровоостанавливающих средств на основе коллагена в сочетании с Na-КМЦ в остром эксперименте *in vivo*. Использовали новые образцы многокомпонентных полимерных губчатых имплантов (МПГИ) (на основе морского коллагена, в разных соотношениях по массе с натриевой солью карбоксиметиллцеллюлозы – Na-КМЦ (15/85, 25/75, 50/50). Оценивали гемостатическую активность (время кровотечения и объем кровопотери) указанных изделий в эксперименте: крысам выполняли лапаротомию и резекцию левой доли печени (серия 1) и нижнего полюса селезенки (серия 2) в коагулометрическом измерении времени свертывания крови доноров-добровольцев. Наименьшие значения оцениваемых показателей (время кровотечния и объем кровопотери) в обоих сериях эксперимента обнаружены в группе 6 с использованием новых образцов МПГИ (Na-КМЦ+коллаген, в соотношении 50/50). Гипотеза об увеличении эффективности использования местных кровоостанавливающих средств при травме паренхиматозных органов за счет разработки комбинированных изделий (а именно на основе Na-КМЦ и коллагена глубоководного кальмара) получила подтверждение в эксперименте, в котором также доказано позитивное влияние внесения коллагена в состав МПГИ на скорость остановки кровотечения (при травме печени наименьший объем кровопотери и время остановки кровотечения — 41 с, а при травме селезенки — 57 с соответственно; $p \leq 0,05$).

Ключевые слова: гемостаз, гемостатические губки, полимеры, эксперимент *in vitro*, кровотечение, коллаген

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Currently, there is a significant number of patients admitted to surgery departments with trauma of the abdominal cavity's parenchymal organs [1, 2]. This category of patients requires special attention, as their injuries can be complicated by massive intra-abdominal bleeding. Despite the advanced diagnostic equipment available at clinics today, including CT, thromboelastography in specialized hospitals, etc., the proportion of fatal liver and spleen trauma cases remains high, at 20 to 60% [3, 4]. Time is of crucial importance: the quicker the patient receives assistance (counting from the moment of injury), the better are his chances of recovery [5].

In such cases, the key goal of assistance is to stop bleeding, which is achievable not only in the context of a surgery but also with the help of a combination of hemostatics [6]. There are various hemorrhage arrest techniques, from the Pringle manoeuvre through atypical resections to suturing the wound [7]. However, currently, the preferred options are those allowing to preserve organs, enabled by the advancements in electrosurgery (coagulators and high-energy equipment forming the final clot), cryosurgery (non equilibrium plasma or cold plasma), multicomponent polymer sponge implants (MPSI), adhesive compositions (sulfacrylate adhesives), etc. [8]. The latter are gels, sponges, plates, powders; the choice of such product's shape depends on the degree of organ damage and its localization, and the possible pattern of surgery (laparotomy, as a rule, since laparoscopic access is used extremely rarely in urgent situations, with unstable hemodynamics a contraindication thereto) [9].

There are many polymers and organic compounds used as base for such products: gelatin, collagen, cellulose derivatives, etc. The respective MPSIs have proven to be effective, and they are common in clinical practice [10]. The relevance of research in this area is underpinned by a large number of publications by national and foreign authors that cover testing of MPSIs in *in vitro* and *in vivo* experiments, the goal of these studies being to find most effective hemostatic that would be highly adhesive and capable of arresting bleeding quickly [11].

This study aimed, in an acute *in vivo* experiment, to comparatively assess the time and volume of bleeding arrested with new collagen sponge hemostatics combined with Na-CMC.

METHODS

The materials used in this study are the new MPSI ("Composite hemostatic sponge," Russian Federation patent application #2023123284 of September 07, 2023; Table 1 below lists characteristics thereof), and hemostatics common in clinical practice.

The study was performed on mature male Wistar rats weighing 200–250 g, under general inhalation anesthesia, in two series (liver and spleen) of 60 animals each, divided into 6 groups as per the number of types of tested MPSIs (Table 1). All surgical interventions were carried out in sterile conditions of the operating unit of the Laboratory of Experimental Surgery and Oncology of the Research Institute of Experimental Medicine of KSMU.

We developed a technique to inflict damage to liver, which included a median laparotomy, liver's left lobe brought out through the wound for marginal resection (10 × 5 × 5 mm) [12]. The injury of the spleen was modeled similarly, with its posterior pole of appropriate dimensions cut off.

The tested sponge, measuring 1.0 × 1.0 cm with a known mass, was applied to the bleeding incision. We registered the volume of blood loss, i.e., how much blood the sponge absorbs, and the time of bleeding. The former (V) was established using the E.M. Levitae gravimetric method, which compares the weight of sterile material before surgery (m1, g) and after (m2, g), when it has siaked up blood. The latter (t, s) was controlled visually and timed with a stopwatch; we lifted the sponge up from the wound every 10 s, and the bleeding was considered arrested when there was no more blood absorbed by it. The animals were removed from the experiment by CO2-induced euthanasia immediately after surgery.

In the context of data processing, we determined the median, 25th and 75th percentiles — Me [25;75] (indicators of descriptive statistics). Due to the small size of the sample on the level of groups (n = 10), we established significance of differences with the help of the Mann-Whitney test, and normality of distribution using the Kolmogorov-Smirnov test, with $p \leq 0.05$, as acceptable for experimental biomedical research. The software used for the purpose was a licensed version of Statistica 13 Pro (Dell Software Company; Round Rock, USA).

RESULTS

According to the results of series 1 experiments (liver injury), hemorrhage was arrested fastest in group 6, where the new MPSI based on marine collagen and Na-CMC was used. In

Table 1. Characteristics of the examined materials and study groups

№	Name	Manufacturer	Composition	Product form
1	Tachocomb	Takeda Austria GmbH, 4020 Linz, Austria	Collagen from horse tendons; riboflavin; lyophilized human fibrinogen; thrombin; aprotinin	Absorbing hemostatic, sponge
2	Surgicel Fibrillar	Ethicon, Johnson & Johnson, USA	Fibers of oxidized and reduced cellulose	Absorbable fibrous hemostatic material
3	Na-CMC	Laboratory of Experimental Surgery and Oncology of the Research Institute of Experimental Medicine of KSMU, AS RS LLC, Kaliningrad, Russia	1% Na-CMC gel	Sponge produced through lyophilic drying of suspension
4	Na-CMC + collagen (85/15)		1% Na-CMC gel 3% suspension of deep-sea squid collagen; 1% Na-CMC gel collagen/Na-CMC ratio, % by weight 15/85	Sponge produced through lyophilic drying of suspension
5	Na-CMC + collagen (75/25)		3% suspension of deep-sea squid collagen; 1% Na-CMC gel collagen/Na-CMC ratio, % by weight 25/75	Sponge produced through lyophilic drying of suspension
6	Na-CMC + collagen (50/50)		3% suspension of deep-sea squid collagen, 1% Na-CMC gel collagen/Na-CMC ratio, % by weight 50/50	Sponge produced through lyophilic drying of suspension

Table 2. Controlled MPSI performance indicators, Me [25;75]

№	Group name	Series 1: liver injury		Series 2: spleen injury	
		Bleeding time, s	Blood loss volume, *m2–m1*, g	Bleeding time, s	Blood loss volume, *m2–m1*, g
1	Tachocomb	93.5 [89.5; 104.75]	0.04 [0.03; 0.05]	105 [101.75; 109.75]	0.024 [0.019; 0.035]
2	Surgicel Fibrillar	85 [83.25; 96.5]	0.02 [0.021; 0.029]	95 [85.5; 101.5]	0.019 [0.017; 0.023]
3	Na-CMC	96 [60.25; 135]	0.019 [0.007; 0.038]	97.5 [85; 126.75]	0.016 [0.01; 0.027]
4	Na-CMC + collagen (85/15)	65 [35.25; 80]	0.006 [0.005; 0.012]	130 [120; 156.75]	0.03 [0.027; 0.033]
5	Na-CMC + collagen (75/25)	97 [80; 122.75]	0.025 [0.017; 0.028]	97 [80; 113.25]	0.015 [0.01; 0.021]
6	Na-CMC + collagen (50/50)	41 [40; 50]	0.01 [0.007; 0.012]	57 [41.25; 70]	0.014 [0.007; 0.024]

that group, the bleeding was stopped 2.3 faster than in group 1, where a collagen plate (commonly used in clinical practice) was used (Table 2, 3). We registered significant differences (twofold and greater) between almost all control groups and group 6, in which the MPSI was 50% collagen, the highest proportion. Group 4, where the MPSI was 15% collagen, also exhibited significant differences with control groups 1 and 2 (sponge plates common in clinical practice).

The bleeding time comparison data given above are supported by the blood loss volume values in the respective study groups (Tables 2, 4). Minimum blood loss was registered in group 6, maximum — in group 3 (MPSI without collagen).

Series 2 (spleen injury) also confirmed efficacy of the sponges developed at KSMU (Table 2, 5, 6). In group 6, the time of bleeding and the volume of blood loss was at least 1.5 times less than in other test groups. The former was significantly different between groups 4 and 6 (Table 5), the latter — significantly different generally (Table 6).

A noteworthy fact is the lack of differences between new MPSI from group 5 and common hemorrhage arresting products used in control groups. However, the blood loss value registered for the group 5 sample differed from that recorded for group 1. It should also be noted that we have also established significant differences among between test groups (both controlled indicators, series 1 and series 2 experiments).

DISCUSSION

There are numerous published papers that present assessments of MPSI based on collagen and cellulose derivatives (usually, oxidized cellulose) that have already been adopted in clinical practice and currently are a standard for comparison, like Tachocomb and Surgicel Fibrillar. Nevertheless, new MPSI are being intensively developed, because the demand for such products is high, and their users are not satisfied with what is commercially available currently [13, 14]. There are solid philosophies dedicated to the design of such medical commodities, each with a certain opinion regarding their composition. In most cases, foreign manufacturers with established reputation on the market of medical products base their MPSIs on animal collagen or fibers of oxidized and reduced cellulose, medical gelatin, etc. [15, 16].

Table 3. Statistical significance of differences, bleeding time, liver injury, *p*

Group name Group №		2	3	4	5	6
		Surgicel Fibrillar	Na-CMC	Na-CMC + collagen (85/15)	Na-CMC + collagen (75/25)	Na-CMC + collagen (50/50)
1	Tachocomb	0.211	0.879	0.037*	0.622522	0.0004*
2	Surgicel Fibrillar		0.791	0.049*	0.363262	0.001*
3	Na-CMC			0.13	1	0.004*
4	Na-CMC + collagen (85/15)				0.129	0.271
5	Na-CMC + collagen (75/25)					0.003*

Note: * — statistically significant differences ($p \leq 0.05$).

Table 4. Statistical significance of differences, blood loss volume, liver injury, *p*

Group name Group №		2	3	4	5	6
		Surgicel Fibrillar	Na-CMC	Na-CMC + collagen (85/15)	Na-CMC + collagen (75/25)	Na-CMC + collagen (50/50)
1	Tachocomb	0.001*	0.053	0.001*	0.003*	0.0002*
2	Surgicel Fibrillar		0.623	0.004*	0.85	0.0002*
3	Na-CMC			0.104	0.677	0.212
4	Na-CMC + collagen (85/15)				0.006	0.623
5	Na-CMC + collagen (75/25)					0.001*

Note: * — statistically significant differences ($p \leq 0.05$).

Table 5. Statistical significance of differences, bleeding time, spleen injury, *p*

Group name Group №		2	3	4	5	6
		Surgicel Fibrillar	Na-CMC	Na-CMC + collagen (85/15)	Na-CMC + collagen (75/25)	Na-CMC + collagen (50/50)
1	Tachocomb	0.064	0.307	0.002*	0.472	0.0002*
2	Surgicel Fibrillar		0.791	0.0005*	0.791	0.0008*
3	Na-CMC			0.045*	0.733	0.003*
4	Na-CMC + collagen (85/15)				0.006*	0.0002*
5	Na-CMC + collagen (75/25)					0.012*

Note: * — statistically significant differences ($p \leq 0.05$).

Table 6. Statistical significance of differences, blood loss volume, spleen injury, *p*

Group name Group №		2	3	4	5	6
		Surgicel Fibrillar	Na-CMC	Na-CMC + collagen (85/15)	Na-CMC + collagen (75/25)	Na-CMC + collagen (50/50)
1	Tachocomb	0.14	0.162	0.623	0.026*	0.054
2	Surgicel Fibrillar		0.623	0.028*	0.344	0.427
3	Na-CMC			0.121	0.571	0.678
4	Na-CMC + collagen (85/15)				0.011*	0.017*
5	Na-CMC + collagen (75/25)					0.791

Note: * — statistically significant differences ($p \leq 0.05$).

Authors of this study accumulated data from the experiments designed to assess properties of MPSI based on marine collagen (publications describing it in this capacity are not freely available) and Na-CMC, which is known to prevent commissures, adhere well and have a pronounced hemostatic effect [17, 18].

Considering the acquired data, we can conclude that effectiveness of an MPSI grows together with concentration of collagen therein, which translates into shorter bleeding time and smaller blood loss. Collagen's hemostatic action has been studied sufficiently; it is assumed to trigger coagulation and blood clot formation. The results of our study confirm veracity of this statement for products based on collagen derived from deep-sea squid. Marine collagen has a number of advantages, including low immunogenicity, which reduces the risk of anaphylactic reactions (possible in case of products based on animal collagen), and high hemostatic efficacy that, in a respective MPSI, is boosted by the porous structure of Na-CMC, which adsorbs the liquid component of blood and thus increases concentration of shaped elements in the sponge-injury contact area.

Such products can be made by national manufacturers of medical commodities; they require no expensive imported raw materials. Subsequent studies of these products (reaction of macroorganism tissues, intraoperative and *in vitro* manipulative properties of MPSI) will allow an assessment of the possibility and prospects of their introduction into the clinical practice of surgical departments.

CONCLUSIONS

The hypothesis tested in this work has the efficacy of MPSI growing due to the addition of collagen (including that of marine origin) to its composition. Based on the resulting data, we can state that the hypothesis was justified: blood loss and bleeding time values were significantly different between control groups and test groups that employed MPSI (six groups, six collagen/Na-CMC ratios). The results of this work are a valid substantiation of further comprehensive testing of the developed MPSIs.

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LOCAL TREATMENT OF A CONTAMINATED SKIN WOUND USING AN ORIGINAL DRUG COMBINATION AND MAGNETIC THERAPY IN AN EXPERIMENT

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Currently, treatment of contaminated skin wounds aggravated by ischemia of superficial soft tissues is a problem that presents certain difficulties. The search for the new ways of treatment and drugs possessing a multidirectional effect is a relevant problem. In this study, we aimed to explore the peculiarities of wound evolution and the effectiveness of the designed combination of medicines and magnetic therapy in a contaminated skin wound case. For the experiment, we divided male Wistar rats into 3 groups and modeled a contaminated skin wound in each of the animals. In the first group, no treatment was performed, in the second, we used the developed combination (benzalkonium chloride, dexpanthenol, pentoxifylline and carboxymethylcellulose sodium salt, combined with magnetic therapy), in the third — ointment with dioxomethyltetrahydropyrimidine + chloramphenicol and magnetic therapy. Planimetry, acid-base balance registration, measurements of microhemocirculation and local temperature of the wound bed underpinned monitoring assessment of the wounds. At the end of the study, the wound area in the second group was 10.7 and 3.7 ($p <$; 0.05) times smaller than in the first and third groups, respectively, and healing rate — 2.6 and 1.3 ($p < 0.05$) times faster. The maximum values of microhemocirculation and the lowest pH were registered in the second group. Thus, combination of drugs and magnetotherapy we designed promoted healing of a contaminated skin wound, which allows recommending this treatment method for further study at the preclinical level.

Keywords: contaminated wound, local wound treatment, benzalkonium chloride, pentoxifylline, wound process

Author contribution: Terekhov AG — study concept and design, analysis of the resulting data, editing; Pankrusheva TA — design of the drug combination, data collection; Chekmareva MS — design of the drug combination, data collection; Turenko EN — collection of material, statistical data processing, analysis of the resulting data, article preparation; Artyushkova EB — collection of material, analysis of the resulting data; Mishina ES — collection of material, statistical data processing, analysis of the resulting data; Grigoryan AYu — analysis of the resulting data, article preparation, editing; Myatechkin AA — analysis of the resulting data, article preparation, editing.

Compliance with the ethical standards: the study was approved by the Ethics Committee of the Kursk State Medical University (Minutes #7 of November 30, 2020). The series of animal experiments, the conditions of their detention met the requirements of the Strasbourg Convention for the Protection of Animal Rights (France, 1986) and GOST 33044-2014 Principles of good laboratory practice.

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МЕСТНОЕ ЛЕЧЕНИЕ КОНТАМИНИРОВАННОЙ КОЖНОЙ РАНЫ ОРИГИНАЛЬНОЙ ЛЕКАРСТВЕННОЙ КОМБИНАЦИЕЙ В СОЧЕТАНИИ С МАГНИТОТЕРАПИЕЙ В ЭКСПЕРИМЕНТЕ

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Лечение контаминированных ран кожи в условиях ишемии поверхностных мягких тканей в современном мире — это проблема, которая представляет определенные трудности. Актуален поиск новых способов и средств лечения, обладающих мультинаправленным действием. Целью исследования было изучить особенности течения раневого процесса и эффективности воздействия на контаминированную кожную рану сочетанного применения разработанной комбинации. Экспериментальную работу проводили на трех группах крыс-самцов породы «Вистар», которым моделировали контаминированную кожную рану. В первой группе лечение не проводили, во второй использовали разработанную комбинацию — бензалкония хлорид, декспантенол, пентоксифиллин и натриевую соль карбоксиметилцеллюлозы, в сочетании с магнитотерапией, в третьей — мазь с диоксометилтетрагидропиримидином + хлорамфениколом и магнитотерапию. Для оценки течения раневого процесса использовали планиметрический метод, определяли кислотно-щелочной баланс, показатели микрогемоциркуляции и локальной температуры раневого ложа. По завершению исследования площадь ран во второй группе была меньше, чем в первой и третьей в 10,7 и 3,7 ($p < 0,05$) раза. Скорость заживления выше во второй группе — в 2,6 и 1,3 ($p < 0,05$) раза. Максимальные показатели микрогемоциркуляции и наименьшие значения pH отмечали во второй группе. Таким образом, сочетанное применение разработанной нами лекарственной комбинации и магнитотерапии благоприятно влияло на процесс заживления контаминированной кожной раны, что позволяет рекомендовать данный способ лечения для дальнейшего изучения на доклиническом уровне.

Ключевые слова: контаминированная рана, местное лечение ран, бензалкония хлорид, пентоксифиллин, раневой процесс

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Currently, treatment of a contaminated wound is a rather complex problem for a medical professional practicing surgery. Chronic wounds associated with diabetes mellitus, chronic arterial insufficiency, translate into disability of patients, cosmetic defects, and also create conditions for the spread of infection, thus increasing the threat of ulcerative necrotic process, subsequent gangrene and amputation [1]. In economically developed countries, the number of limb amputations varies from 13.7 to 32.3 for every 100,000 people, with 50% of amputees dying within the first year thereafter, which underpins the urgency of this problem [2, 3]. This group of patients needs inpatient treatment. Open wounds require dressings that prevent entry of microorganisms thereinto, and contain no components that have toxic, allergic, mutagenic, and carcinogenic effects [4]. Considering the methods of treatment, a practitioner should look for shorter healing time, prevention of complications, and scar tissue esthetics. These criteria substantiate the search for new techniques, development of drug combinations and a balance between medicinal and physiotherapeutic parts of wound treatment [4].

Thus, the question of creating a new multicomponent drug combination that will meet all the above requirements takes priority. Sodium salt of carboxymethylcellulose (Na-CMC), on which the active substances are immobilized, can be the basis thereof. As reported in the literature, Na-CMC is the base for films that accelerate formation and maturation of new tissue, influence fibrillogenesis, and also markedly stimulate reparative processes in the infected skin wounds [5]. Na-CMC-based gels are used to prevent intraoperative drying of peritoneum and formation of postoperative commissures in the context of operations on organs with a serous coating [6].

It is feasible to augment the combination with a component that enhances skin regeneration. One of these is dexpanthenol; this drug, applied topically, turns into pantothenic acid, which, in turn, is part of coenzyme A. All oxidoreductases require a coenzyme: redox processes are impossible without it. Dexpanthenol enhances epidermal differentiation and proliferation of dermal fibroblasts, thereby supporting skin regeneration [7]. Therefore, there have been designed various topical preparations containing this compound, widely used in dermatology. Topically, dexpanthenol is also recommended in cases of small and superficial wounds [8].

The preferred antiseptic should be bactericidal, since pathogenic microflora is less likely to grow resistant thereto; one of the proven agents of this kind is benzalkonium chloride. It reduces surface tension between two media and attracts negatively charged particles, thus disrupting integrity of the cell membranes, upsetting denaturation of intracellular proteins, and disordering metabolic processes in the cells, which triggers release of vital elements into intercellular space and ultimately eliminates the microorganisms [9].

Since we are considering healing of a contaminated wound, it seems promising to complete the combination with a component that improves microcirculation in the tissues, such as pentoxifylline. Previous studies confirm that pentoxifylline improves blood's rheological parameters by reducing the viscosity plasma and whole blood, increasing the elasticity of erythrocyte membranes and suppressing erythrocyte aggregation, and reducing platelet aggregation. The compound also possesses anti-inflammatory and antioxidant properties [10]. To boost healing, many researchers recommend extending the treatment protocol with physical factors, such as magnetotherapy, since an external magnetic field supports targeted delivery of the therapeutic nanocomplex and helps maintain concentration of the drug in the wound at the optimal level [10, 11].

Therefore, this study aimed to investigate the specifics of the wound process and the efficacy of the combination of benzalkonium chloride, dexpanthenol, pentoxifylline, and magnetic therapy on contaminated skin.

METHODS

The study included *in vivo* experiments on 90 white male Wistar rats. The animals were allocated into 3 groups ($n = 30$). The weight of each rat was 180.0 ± 20.0 g. All animals received inhalation anesthesia in a sterile operating room at the Laboratory of Experimental Surgery and Oncology of the Experimental Medicine Research Institute, and had a contaminated skin wound modeled (ischemic conditions) by our proprietary method (patent decision 2023124868/14, invention "Method for modeling a skin wound in ischemic conditions").

Would modeling required access to the femoral neurovascular bundle on the medial surface of the thigh under inguinal ligament. Using 4/0 catgut, we ligated a.femoralis and resected 1/3 of its trunk distally from the inguinal ligament. Seven days 7 days after resection, on a shaved patch of skin, after applying an antisetic solution and hydrotreating the field with 0.9% NaCl solution (5 ml), we excised a 14 mm round skin flap (down to the superficial fascia) in the middle third of the anterolateral surface of the thigh. After hemostasis, the wound was covered with an aseptic dressing. For 4 days, the wound was not treated, dressed with a Cosmopor bandage with the absorbent pad removed, which created conditions for its contamination. To standardize the treatment process, a special protective collar for rats was put on animals. The rats were kept in individual boxes (cages) to prevent contact between them, and ate the same standard diet. The bedding was replaced once a day in all cages. On the 5th day after the excision, we started treatment, which was when the experiment was considered launched. The presence of a contaminated wound formed under ischemic conditions was confirmed by microbiological examination and laser Doppler fluorometry of the affected limb.

Study groups:

Group 1 — control group, no treatment;

Group 2 — treatment with a combination of benzalkonium chloride + dexpanthenol + pentoxifylline (topically) + NaCMC + magnetic therapy;

Group 3 — treatment with an dioxomethyltetrahydropyrimidine ointment + chloramphenicol ointment combined with magnetic therapy.

According to the register of medicines, dioxomethyltetrahydropyrimidine + chloramphenicol ointment has anti-inflammatory and antimicrobial effects; it combats gram-positive and gram-negative microorganisms, easily penetrates deep into the tissues without damaging biological membranes, and stimulates regeneration. Its antibacterial effect persists in the presence of pus and necrotic masses. This ointment is widely used in outpatient practice.

Combinations of drugs and physiotherapeutic methods of treatment:

1) benzalkonium chloride 0.02 g + dexpanthenol 5 g + 2% pentoxifylline solution up to 100 g (topically) + NaCMC 4.0 g and magnetotherapy;

2) dioxomethyltetrahydropyrimidine ointment + chloramphenicol and magnetotherapy.

Second group received 0.5 ml of the respective gel to the wound and magnetotherapy in the given mode; in the third group, it was 0.5 ml of the dioxomethyltetrahydropyrimidine ointment + chloramphenicol and magnetotherapy. For the

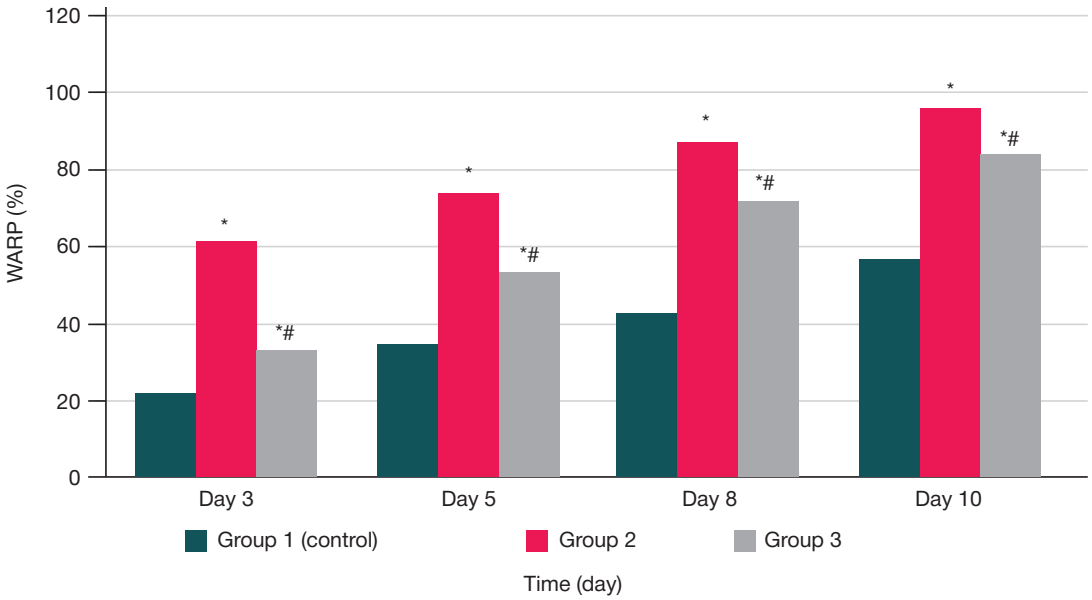

Fig. 1. Wound area reduction percentage (%), Me (25; 75). * — $p < 0.05$ in comparison of group 1 (control) and other groups; # — $p < 0.05$ in comparison of group 2 and group 3.

latter, we used Milta-F-8-01 (Binom; Russia) (GOST25052-87) magnetic, IR, and laser therapy device in the magnetotherapy mode. The frequencies used were 80, 150, 300, 600, 1500, 5000 Hz; power — 50 MW; session duration — 6 min (1 min at each frequency), conducted once a day.

The treatment protocol implied daily dressings in sterile conditions for 10 days, the bandages carrying the above combinations.

We used Lesion Meter planimetry software to monitor the progress.

The percentage of wound area reduction was calculated from the initial size by the following formula:

$$WARP = \frac{S_0 - S}{S_0} \times 100\%,$$

where WARP is the wound area reduction percentage, S_0 the initial average wound area at the beginning of treatment, mm^2, and S the average wound area at the time of measurement, mm^2.

The rate of wound healing was calculated by the following formula:

$$HR = \frac{WARP_1 - WARP_0}{T},$$

where HR is the healing rate, $WARP_1$ is the wound area reduction percentage (compared to the initial size) at the time of measurement, $WARP_0$ the wound area reduction percentage at the previous measurement, and T the number of days between measurements.

To monitor microcirculation in the wound and the surrounding tissue, we employed laser Doppler flowmetry (LDF), with LDF100C laser Doppler flow module (Biopac system Inc.; USA) and TSD-144 probe taking measurements, and Acq Knowledge 4.2 for MP150 doing the processing. The acid-base balance was determined by recording the pH values on the wound surface using a PH98110 pH meter (Kelilong; China), and local temperature was taken with the help of a WF-5000 infrared thermometer (B.Well; Switzerland) [12, 13].

The results of the experimental study were recorded on the 1st, 3rd, 5th, 8th, and 10th day. For statistical processing thereof, we used Microsoft Excel 2014 and Statistica 13.0 software. Quantitative attributes were given as median, 25th and 75th percentiles (Me (25; 75)). For statistical analysis, we applied the Kruskal–Wallis test to the results, and compared the mean ranks by groups. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Planimetry showed that on the first day, WARP was similar in all three groups studied. It was gradually decreasing through the experiment; in group 2, WARP was the largest among the three groups already on the 3rd day, with the differences being significant (Fig. 1). Overall, on the 3rd day, the figures were as follows: group 1 — (21.26 (20.6; 25.19) %), group 2 — (61.54 (57.47; 65.77) %), group 3 — (33.18 (30.6; 36.36) %). Thus, in absolute values, WARP in group 2 was 2.9 times greater than in group 1 and 1.8 times greater than in group 3. On the 5th day, the difference, remaining significant, was as follows: group 1 — (73.5 (76.85; 81.41) %), which is 2.1 times more than in group 1 (34.69 (28.13; 39.87) %) and 1.4 times more than in group 3 (53.33 (47.85; 55.77) %). By the end of the experiment, on the 10th day, WARP in group 2 was (95.74 (89.45; 99.92) %), in group 1 — (56.22 (54.53; 61.91) %), in group 3 — (84.59 (73.35; 86.78) %); the differences were significant, with the values in group 2 1.7 and 1.1 times greater than in group 1 and group 3, respectively.

The data in Table 1 indicate that during the first 3 days, healing rate in group 2 was significantly higher than in group 1 and group 3 (2.2-fold and 1.4-fold, respectively). Group 2 keeps its leadership during days 5 through 8, with healing rate there 2.8 and 1.3 times greater than in groups 1 and 3, respectively. By the end of the experiment, during days 8 through 10, the differences, still significant, were 1.9 times and 1.2 times (group 2 vs. group 1 and group 3, respectively).

Weighted average LDF values (surfaces of the wounds) of group 2 were significantly different from those of groups 2 and 3 on the 3rd, 5th, 8th, and 10th day of the experiment (Fig. 2). In terms of perfusion units (p.u.), on the 3rd day, the values in group 2 were (304.74 (288.21; 320.1)), which is 1.2 and 1.03 times more than in groups 1 (253.18 (245.39; 260.27) p.u.) and the 3 (293.77 (278.51; 307.01) p.u.). Data for the 5th day: group 1 (269.26 (263.15; 275.79) p.u.)), group 2 (371.69 (366.58; 377.17) p.u.)), group 3 (341.07 (334.61; 345.88) p.u.)). Thus, the values registered in group 2 are 1.4 and 1.08 times higher

Table 1. Dynamics of wound healing in the treated experimental animals, Me (25; 75)

Group	Healing rate, %/day			
	Days 1–3	Days 3–5	Days 5–8	Days 8–10
	n = 24	*n* = 18	*n* = 12	*n* = 6
Group 1 (control)	9.05 (7.75; 2.66)	5.17 (3.66; 7.93)	2.61 (1.90; 3.19)	3.52 (2.74; 3.88)
Group 2	20.38 (18.80; 22.67)*	15.99 (11.99; 16.11)*	8.70 (6.98; 9.46)*	7.02 (4.91; 8.2)*
Group 3	14.22 (11.39; 15.32)*,**	11.96 (6.73; 11.38)*,**	6.66 (3.69; 8.56)*,**	5.91 (3.85; 9.14)*,**

Note: * — $p < 0.05$ in comparison of group 1 (control) and other groups; ** — $p < 0.05$ in comparison of group 2 and group 3.

than in groups 2 and 3. On the 8th day, the difference in the LDF value between group 1 (289.18 (284.97; 292.76) p.u.) and group 2 (461.17 (457.33; 463.07) p.u.) was 1.6 times, between group 1 and group 3 (403.84 (399.66; 407.39) p.u.) — 1.1 times. By the end of the experiment, on the 10th day, the value in group 2 (505.11 (499.29; 511.71) p.u.) was significantly higher than in group 1 (301.45 (296.23; 307.01) p.u.) and group 3 (436.93 (431.59; 443.34) p.u.), by 1.7 and 1.1 times, respectively.

The analysis of the wound acid-base balance data reveals that on days 3, 5, 8, and 10, the respective value in group 2 was significantly lower than in groups 1 and 3 ($p < 0.05$) (Table 2). On the 3rd day, the difference was 1.2 times 1.1 times (group 2 vs. group 1 and group 2 vs. group 3, respectively). The dynamics persisted through day 5. In comparison of the groups, the lowest pH values were registered in group 2, the greatest significant difference recorded on the 10th day: by 1.4 and 1.3 times for groups 1 and 3, respectively.

Wound bed thermometry revealed no differences between the groups on the 1st day of the experiment (Fig. 3). On the 3rd day of treatment, local temperature was the lowest in groups 2 (34.15 (33.6; 34.5) °C) and 3 (33.95 (33.7; 34.3) °C); the difference with the control group (35.25 (35.1; 36.05) °C) was significant, and equaled 1.03 times for both groups. On the 8th day, the difference between group 1 (37.85 (37.5; 38.8) °C) and groups 2 and 3 was still 1.03 times: (36.55 (36.45; 36.8) °C) in group 2, and (36.83 (35.45; 37.3) °C) in group 3, by 1.03 times. Thus, the progress in the control group was the weakest. Moreover, on the 10th day, the difference increased to 1.2 times compared to the 1st day (38.92 (38.3; 39.3) °C vs. (33.75 (33.2; 34.3 °C).

DISCUSSION

Planimetry data shows that significantly higher values were registered in group 2 on all days of the experiment. As for the healing rate, on days 1 through 5, this indicator was greater in group 2 than in groups 1 and 3 by 2.6 and 1.4 times, respectively. LDF values were also highest in group 2: 1.3 and 1.2 times higher than in groups 1 and 3, respectively, which means the wounds in group 2 had the best loacal blood microcirculation. In terms of pH, the values in group 2 were significantly better than in groups 1 and 3 on days 3 through 10, which indicated development of an acidic environment that adversely affects pathogenic microorganisms. Local temperature was significantly lowest in groups 2 and 3, compared to the control, on days 8 and 10; moreover, in group 1, wound bed temperature was steadily increasing, which may have indicated a pronounced inflammatory process.

Reports by other authors are consistent with our findings: the components we used in the combination effectively accelerate the processes associated with wound healing.

Thus, topical pentoxifylline improved local blood flow in the injured tissue, which boosted healing [14]. It was also proven effective against burn wounds [15]. A randomized prospective clinical trial confirmed beneficial effects of a dexpanthenol ointment applied to skin damaged as a result of fractional ablative CO_2 laser resurfacing. The authors found that in dry skin, dexpanthenol can compensate, to some extent, for low hydration by increasing the water content and producing a positive effect on the molecular mobility of lipid layers and stratum corneum proteins [16]. A number of authors have investigated the physico-chemical properties and therapeutic effect of benzalkonium chloride. This antiseptic was found to possess a pronounced antimicrobial powers not only against pathogenic bacteria, but also *Candida* fungi [17]. Another group investigated the effect of benzalkonium chloride carried by polyethylene oxide on the purulent-inflammatory process in soft tissues; the results confirmed that this antiseptic accelerates healing rate of the skin defect in the first phase of the wound process [18].

There have also been conducted studies looking into the benefits of magnetotherapy in the context of would healing. One has established that a pulsed electromagnetic field applied to patients with diabetic angiopathy accelerated wound healing by 1.5 times [19]. Another reported a positive effect of therapeutic magnetic resonance on the healing of trophic

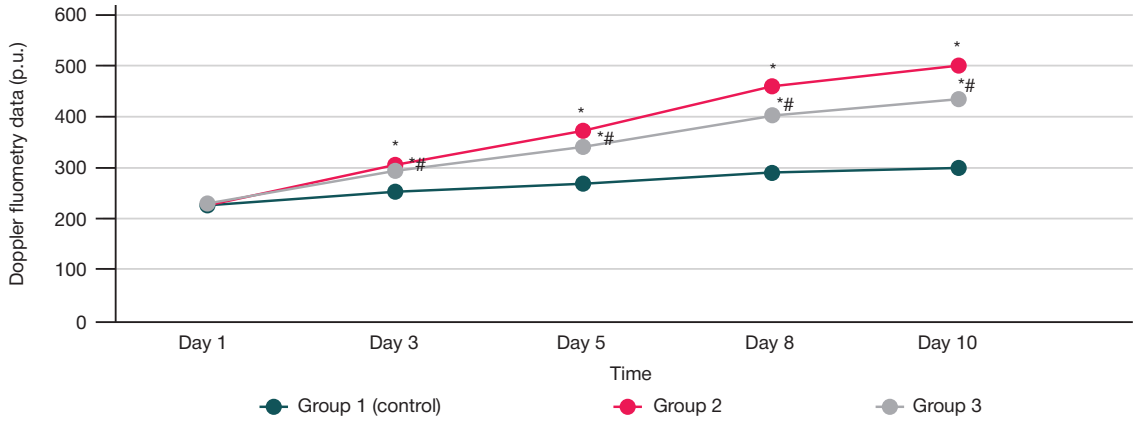

Fig. 2. Laser Doppler flowmetry dynamics (p.u.), Me (25; 75). * — $p < 0.05$ in comparison of group 1 (control) and other groups; # — $p < 0.05$ in comparison of group 2 and group 3

Table 2. Wound pH changes, Me (25; 75)

Group	Day 1 *n* = 30	Day 3 *n* = 24	Day 5 *n* = 18	Day 8 *n* = 12	Day 10 *n* = 6
Group 1 (control)	7.7 (7.54; 7.91)	7.54 (7.38; 7.71)	7.22 (7.18; 7.36)	7.275 (7.18; 7.36)	7.22 (7.11; 7.32)
Group 2	7.56 (7.02; 7.45)	6.5 (6.55; 6.83)*	6.28 (6.33; 6.512)*	5.42 (5.55; 6.245)*	5.01 (4.82; 5.95) *
Group 3	7.63 (7.54; 7.99)	7.33 (7.20; 7.37)#	7.27 (6.93; 7.52)#	6.83 (6.55; 6.935)#	6.58 (6.43; 6.84) *,#

Note: * — $p < 0.05$ in comparison of group 1 (control) and other groups; # — $p < 0.05$ in comparison of group 2 and group 3.

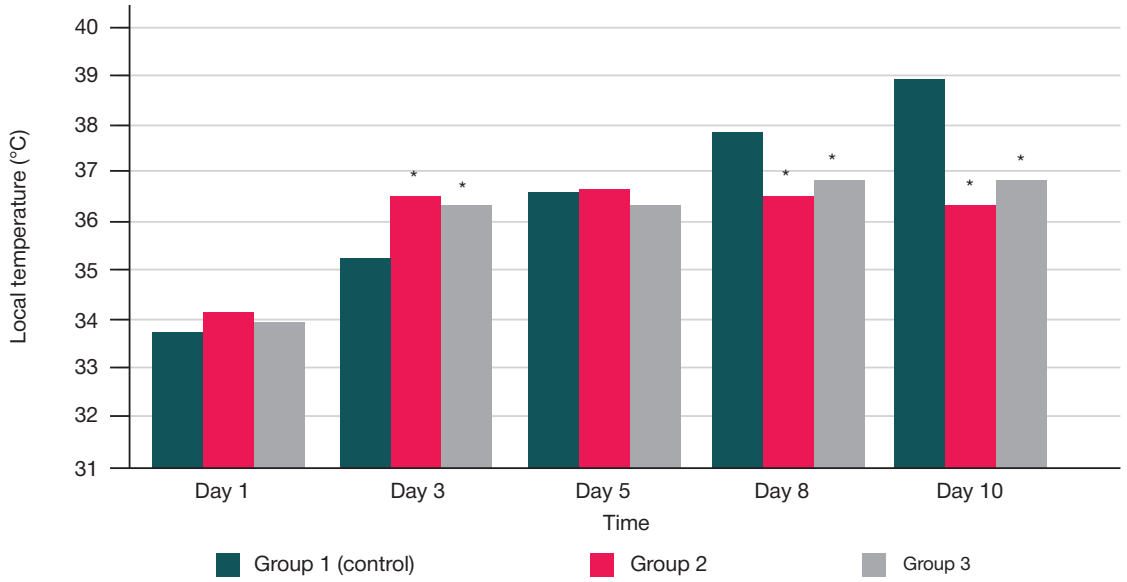

Fig. 3. Local wound temperature dynamics (°C), Me (25; 75); * — $p < 0.05$ in comparison of group 1 (control) and other groups; # — $p < 0.05$ in comparison of group 2 and group 3

ulcers, which took 44 days in the experimental group and 97 days in the control group [20].

CONCLUSIONS

Based on the planimetry, wound microhemocirculation, acid-base balance, wound bed thermometry data collected in this study, we can conclude that the woulds healed in the most efficient way in group 2, where the treatment was by the method we suggested. Therefore, in the context of treatment of contaminated skin wounds, we can recommend further research of the combination of benzalkonium chloride + dexpanthenol + NaCMC + pentoxifylline + magnetotherapy.

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FAMILIAL CASE OF INHERITED HUMAN HERPESVIRUS 6A WITH PHYLOGENETIC ASSESSMENT

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The paper reports a familial case of HHV-6A chromosomal integration being an important and relevant issue of genetics and medicine. The study was aimed to test the hypothesis of HHV-6A chromosomal integration and vertical transmission in patient with persistent virus detection during recurrent respiratory diseases and the asymptomatic period when there were no health complaints. Sequencing of the patient's father genome DNA was performed, and a phylogenetic tree was constructed by aligning 270 HHV-6A/B genome assemblies from the GenBank database. As a result, a familial case of ciHHV-6A transmission was identified. It was found that the detected ciHHV-6A observed on the phylogenetic tree was closely related to other two chromosomally integrated HHV-6A sequences reported by Moscow researchers. The study confirmed HHV-6A chromosomal integration. Further precise chromosome mapping of ciHHV-6A would be useful in terms of excluding probable somatic disorders associated with the chromosome structure alteration following HHV-6, particularly HHV-6A, integration, as well as for identification of insertion sites specific for various geographic locations.

Keywords: human herpesvirus 6A/B (HHV-6A/B), chromosomal integration, ciHHV-6A/B, inherited herpesvirus, phylogenetics

Author contribution: Goleva OV, Babachenko IV, Tian NS — study planning, data acquisition, analysis and interpretation, manuscript draft; Danilov LG — bioinformatics analysis, search for analytical papers; Kusakin AV — study planning, literature review, data acquisition, analysis and interpretation, bioinformatics analysis, constructing a phylogenetic tree, manuscript draft; Eismont YuA, Chukhlovin AB — study planning, data acquisition, analysis and interpretation; Krylov AV — data acquisition, analysis and interpretation; Glotov OS — research supervision, data analysis and interpretation, manuscript draft.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia (protocol № 107 dated November 27, 2018) and conducted in accordance with the latest edition of the Declaration of Helsinki. Patients and their legal representatives submitted the informed consent to the study participation.

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СЕМЕЙНЫЙ СЛУЧАЙ НАСЛЕДУЕМОЙ ХРОМОСОМНОЙ ИНТЕГРАЦИИ ВГЧ-6А С ПРОВЕДЕНИЕМ ФИЛОГЕНЕТИЧЕСКОГО АНАЛИЗА

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В статье рассмотрен семейный случай хромосомной интеграции ВГЧ-6А, которая является важной и актуальной темой в области генетики и медицины. Целью исследования было проверить гипотезу о хромосомной интеграции ВГЧ-6А и его вертикальной передаче у пациента с длительным обнаружением вируса во время рекуррентных респираторных заболеваний, а также в бессимптомный период, при отсутствии жалоб на здоровье. Проведено секвенирование геномной ДНК отца пациента, построено филогенетическое дерево путем выравнивания 270 геномных сборок ВГЧ-6А/В из базы данных GenBank. В результате исследования установлен семейный случай передачи хиВГЧ-6А. Показано, что обнаруженный хиВГЧ-6А, наблюдаемый на филогенетическом древе, находится в тесном контакте с двумя другими хромосомно-интегрированными последовательностями ВГЧ-6А, о которых сообщали московские исследователи. Исследование подтвердило хромосомную интеграцию ВГЧ-6А. Дальнейшее точное хромосомное картирование хиВГЧ-6А/В было бы полезно для исключения вероятных соматических заболеваний, связанных с изменением структуры хромосом при интеграции ВГЧ-6, в частности ВГЧ-6А, а также для идентификации участков инсерции, специфичных для различных географических точек.

Ключевые слова: вирус герпеса человека 6А/В (ВГЧ-6А/В), хромосомная интеграция, хиВГЧ-6А/В, унаследованный герпесвирус, филогенетика

Вклад авторов: О. В. Голева, И. В. Бабаченко, Н. С. Тян — планирование исследования, сбор, анализ, интерпретация данных, подготовка черновика рукописи; Л. Г. Данилов — проведение биоинформатического анализа, поиск аналитических материалов; А. В. Кусакин — планирование исследования, анализ литературы, сбор, анализ, интерпретация данных, проведение биоинформатического анализа, построение филогенетического древа, подготовка черновика рукописи; Ю. А. Эйсмонт, А. Б. Чухловин — планирование исследования, сбор, анализ, интерпретация данных; А. В. Крылов — сбор, анализ, интерпретация данных; О. В. Глотов — курирование исследования, анализ, интерпретация данных, подготовка черновика рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБУ ДНКЦИБ ФМБА России (протокол № 107 от 27 ноября 2018 г.) и выполнено согласно Хельсинской декларации последнего пересмотра. Получено письменное информированное согласие пациентов и их законных представителей на участие в исследовании.

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Human betaherpesvirus 6A/B (HHV-6A/B) is widely spread in human population. In 1986, the research team of the laboratory at the National Cancer Institute (USA) isolated the virus from patients with lymphoproliferative diseases and identified it as human B-lymphotropic virus, however, later its affinity for T cells and belonging to the family *Herpesviridae*, subfamily *Betaherpesvirinae*, genus *Roseolovirus*, were determined [1]. The virus is primarily transmitted through contact with saliva or less often by airborne droplets, sexual contact, and transplanted organs. $CD4^+$ T cells are the main target cells for the virus. HHV-6 enters the cells via receptor-mediated endocytosis followed by virus replication. After primary infection viral DNA persists in mononuclear peripheral blood cells [2, 3]. HHV-6A/B can trigger immunosuppression and chronic autoimmune processes [4].

In 2012, International Committee on Taxonomy of Viruses (ICTV) ratified HHV-6A division into two distinct taxonomic variants: HHV-6A and HHV-6B [5, 6]. Despite the fact that the genomes of these viruses demonstrate 90% homology, the viruses show phenotypic differences, are tropic for different cellular receptors, and in the majority of cases have different clinical manifestations [7]. HHV-6A is a less explored virus, it is acquired later in life and more often detected in immunocompromised individuals. It is assumed that this virus is associated with such neurodegenerative disorder as Alzheimer's disease [3, 8]. HHV-6B is common everywhere, more than 90% of the population get infected during the first three years of life, while reactivation can occur at any age [3]. The viruses show different tropism against immunocompetent cells. Thus, HHV-6A uses CD46 receptors for cell entry, it is capable of affecting T helper cells, cytotoxic T cells, and natural killers. By contrast, HHV-6B uses CD134 receptors and fails to persist in cytotoxic T cells [9, 10].

HHV-6A/B genome consists of a double-stranded DNA with an average length of about 160 kbps. It is noteworthy that the genome of HHV-6A is shorter than that of HHV-6B, it is about 159 kbps vs. 162 kbps, respectively [11]. The majority of genes are located in the unique segment flanked by direct repeats (DR). DRs, in turn, are surrounded by pac1 and pac2 being the cis-acting packaging signals [11, 12]. The number of open reading frames (ORF) depends on the virus type (A/B) and the detection method. A total of 115–119 ORFs were earlier predicted based on the sequence [11, 13], however, the researchers managed to identify 268 ORFs in HHV-6A and 216 in HHV-6B using advanced Ribo-seq and RNA-seq methods [8]. The average sequence similarity of HHV-6A and HHV-6B is 90%. U39 and U48 that encode gB and gH envelope glycoproteins, respectively, are the most conservative genes. Their nucleotide sequences show 94% similarity, and amino acid sequences show 96% similarity [11, 14]. Moreover, the most variable genes that are located close to the genome termini primarily encode proteins that are likely to be involved in immunomodulation, signaling (chemotaxis), and viral entry [12].

Polymerase chain reaction (PCR) is considered to be the main method of HHV-6A/B diagnosis, however, to date no clear boundary between identification of latent and active viral infection based on PCR results has been determined. Absence of HHV-6A/B DNA in blood plasma or serum does not mean that there is no persistent virus in low concentrations in the tissues (for example, in the heart, thyroid gland, brain). Detection of specific IgM and IgG antibodies in blood serum is also of some diagnostic significance [16]. The researchers have proposed the test systems considering different reading frames for HHV-6A (U11, p100) and HHV-6B (101K) [17].

HHV-6 capability of integration into subtelomeric region of the cellular chromosome was found in 1993 [18]. Today, it is known that viral integration most often occurs in the telomeric regions of chromosomes 1q, 6q, 9q, 10q, 11p, 17p, 18p, 19q, 22q, Xp, however, the mechanisms are poorly understood [19–23]. HHV-6 integration into the germ cell genome enables transmission of the virus to the next generations and formation of the inherited chromosomally integrated HHV-6A/B (inherited ciHHV-6A/B) in accordance with the Mendel's laws [24]. CiHHV-6A/B can be also transmitted with the transplanted cells, organs, and tissues. CiHHV-6A/B abundance varies between 0.2% in Japan, 0.6% in Canada and 1–3% in Europe, it depends on geographic factors and the assessed population of patients [25, 26].

The cases of integrated ciHHV-6A/B reactivation up to clinically manifested forms in individuals with immunodeficient conditions and pregnancy have been reported [2, 27, 28]. Reactivation of the chromosomally integrated virus during pregnancy can result in the increased risk of spontaneous abortion [29]. The British study conducted in 2020 showed that women with fetuses infected with ciHHV-6A/B had a 2.5–3 times higher risk of preeclampsia [30]. Biological and medical effects of HHV-6A and HHV-6B chromosomal integration are currently being studied. For example, telomeres linked to endogenous HHV-6A/B are often prone to sudden deletions, which lead to telomere shortening. As a result, premature cell ageing and impaired tissue homeostasis are observed [31–33]. Genome instability can cause cancer.

The study was aimed to test the hypothesis of the HHV-6A chromosomal integration and vertical transmission in patient with persistent virus detection during recurrent respiratory diseases and the asymptomatic period when there were no health complaints.

METHODS

Five family members, mother (36 years old), father (39 years old), three sons (4 years, 6 years, and 14 years old), were the research objects. The family lived in the town of Kirishi (Leningrad region).

Nucleic acid isolation and HHV-6A/B detection

Nasopharyngeal smears and venous blood were collected during the study for further molecular genetic tests and enzyme-linked immunoassay. Specific fragments of nucleic acids of influenza viruses A and B, respiratory syncytial virus, type 1–4 parainfluenza viruses, seasonal coronaviruses, metapneumovirus, rhinoviruses, as well as DNA of group B, C, E adenoviruses and bocaviruses were detected in the nasopharyngeal smears using AmpliSens Influenzavirus A/B-FL and AmpliSens ARVI-screen-FL kits (Rospotrebnadzor; Russia) for multiplex PCR with fluorescent hybridization detection of amplification products. DNA of Epstein–Barr virus (EBV), HHV-6A/B and cytomegalovirus (CMV) was detected in blood and oropharyngeal mucosal smears by real-time PCR (RT-PCR) using AmpliSens EBV/CMV/HHV6-screen-FL kit (Rospotrebnadzor; Russia). The HHV-6A/B viral load in the studied biomaterials was assessed within the range of 22–38 amplification cycles (Ct) and expressed in genome equivalents per 1 mL (gEq/mL) of native sample after preanalytical processing. The results obtained within the range of 35 cycles (10^3–10^4 gEq/mL) were considered to be of diagnostic significance. Venous blood collected into K2-EDTA blood sampling tubes was used to extract DNA. Oropharyngeal smears were placed into Transport Medium with Mucolytic Agent (ILS; Russia). DNA was extracted from venous blood

Table 1. Laboratory markers of herpesvirus infections at admission

Biomaterial	Markers of herpesvirus infection	Patient assessment results	
		S.T., 4 years old	S.A., 6 years old
Blood	EBV DNA EBV IgG (VCA)	Positive Positive	Positive Positive
Blood	HHV-6A DNA	Negative	105*
Oropharyngeal smear	HHV-6A DNA	Negative	104*
Blood	HHV-6B DNA	103*	Negative
Oropharyngeal smear	HHV-6B DNA	104*	Negative
Blood	HHV-6 IgG	Negative	6.9**

Note: * — gEq/mL; ** — AU.

using MagNa Pure automated nucleic acid extraction system (Roche; Switzerland) by standard sample preparation method. Biomaterial from the oropharyngeal smear was purified using RealBest Extraction 100 kit (Vector-Best; Russia). Sample preparation and extraction were carried out in accordance with the manufacturers' instructions. Extraction was controlled with NanoStar spectrophotometer (BMG; Germany). The extracted material was quantified using Quantus fluorimeter (Promega; USA).

The standard *GAPDH* cellular gene was used to test the extracted sample for the presence of DNA and its quality [34]. Amplification was performed in CFX-96 PCR system (BioRad; USA) using qPCRmix-HS kit (Evrogen; Russia).

IgM and IgG antibodies against the listed above herpesviruses were detected by qualitative enzyme-linked immunosorbent assay (ELISA) using VectoEBV-VCA-IgM/G and VectoCMV-IgM/G kits (Vector-Best; Russia) and semi-qualitative ELISA using the HHV-6-IgM/G-ELISA-BEST kit (Vector-Best; Russia) in the open type Lazurite unit (Dynex Technologies Inc.; USA) within the framework of standard laboratory testing. The results were represented by positivity rate (PR) expressed in arbitrary units (AU) according to the test system manufacturer's instructions.

The semen sample collected from the father was used as supplementary material. Spermatozoa were obtained by density gradient centrifugation using SupraSperm System (ORIGIO; USA) for extraction of viable sperm. DNA was isolated from spermatozoa by phenol chloroform extraction. The quality of isolated DNA was estimated using 4200 TapeStation System and Genomic DNA ScreenTape kit (Agilent Technologies; USA), concentration was measured using QuantiFluor dsDNA System (Promega; USA).

Other laboratory tests

Standard diagnostic tests were supplemented by differentiation of HHV-6A/B variants using the reported primers [34]. Alignment of primers to the HHV-6 reference sequences was checked using the BLAST tool (NCBI; USA):

HHV-6A/B FP: 5'- GACAATCACATGCCTGGATAATG-3';
HHV-6A RP, 5'- TGGTAATGTAATTGTGTGTTGTTTTA-3';
HHV-6B RP, 5'- TGGTAATGTAAGTGTGCGTTATTTTC-3';
HHV-6 probe, 5'-FAM- AGCAGCTGGCGAAAGCTGTGC-TAMRA-3'.

NGS library preparation

The sequencing libraries were prepared for two instruments in order to obtain long and short reads. Long reads were acquired using MinION system (Oxford Nanopore Technologies; UK). Libraries were prepared in accordance with the whole-genome sequencing protocol using SQK-LSK109 sample preparation kit (Oxford Nanopore Technologies; UK) and NEBNext module (New England Biolabs Inc.; USA) for preparation of Oxford Nanopore Technologies libraries (NEBNext). Short reads were acquired by sequencing in the MGISEQ 2000 system (MGI Tech Co.; China). Libraries were prepared in accordance with the guidelines [35].

The quality of resulting libraries was assessed using D1000 ScreenTape and High Sensitivity D1000 ScreenTape kits (Agilent Technologies; USA); concentration was measured with Quantus fluorimeter using QuantiFluor dsDNA System kit (Promega; USA).

DNA sequencing

To perform whole-genome sequencing in the MinION system, the R10 (FLO-MIN111) flow cell for nanopore sequencing (Agilent Technologies; USA) was used.

Whole-genome sequencing in the MGISEQ 2000 system was performed using the DNBSEQ-G400 CoolMPS High-throughput Sequencing Set (PE100, 320 G) (MGI Tech Co.; China). One lane was selected for whole-genome sequencing.

Table 2. Laboratory markers of herpesvirus infections obtained during re-examination eight months later

Biomaterial	Markers of herpesvirus infection	Patient assessment results	
		S.T., 4 years old	S.A., 6 years old
Blood	EBV DNA	Negative	Negative
Blood	HHV-6A DNA	Negative	106*
Oropharyngeal smear	HHV-6A DNA	Negative	104*
Blood	HHV-6B DNA	Negative	Negative
Oropharyngeal smear	HHV-6B DNA	Negative	Negative
Blood	HHV-6 IgG	5.4**	6.1**

Note: * — gEq/mL; ** — AU.

Table 3. Laboratory markers of herpesvirus infection in other family members

Biomaterial	Markers of herpesvirus infection	Patient assessment results		
		S. A., 14 years old	Mother, 36 years old	Father, 39 years old
Blood	HHV-6A DNA	106*	Negative	106*
Oropharyngeal smear	HHV-6A DNA	104*	Negative	105*
Semen	HHV-6A DNA	–	–	106*
Blood	HHV-6B DNA	Negative	Negative	Negative
Oropharyngeal smear	HHV-6B DNA	Negative	Negative	Negative

Note: * — gEq/mL.

Genome assembly

The data obtained from the Nanopore platform were used for viral genome assembly. Genome was assembled using the customized assembly line: the herpes virus-associated reads were extracted with the Cookiecutter tool [36] using Moscow strain (GenBank ID: MK630134, MK630133) as reference [37], since it was characterized by larger depth (500x). Later only a fragment of gene *gB* (U39) was used in the study, which was completely assembled in these sequences. The reads were assembled with the SPAdes tool [38]; the assembled contigs were configured manually by searching for complete reference sequence in BLAST [39].

Phylogenetic analysis

The glycoprotein B (*gB*, U39) HHV-6A gene (Gene ID: 1487917) nucleotide sequence was used for phylogenetic analysis. All sequences of 270 herpes virus assemblies (both 6A and 6B) available from the GenBank database were included in the analysis. MAFFT v7.505 algorithm with Kimura 1 parameter substitution model were used for sequence alignment [40]. Then the resulting alignments were arranged to construct the tree using the Neighbor-Joining method (Jukes-Cantor, Bootstrap resampling = 100) [41].

RESULTS

In December 2018, the child S. A., 6 years old, with his brother S. T., 4 years old, were admitted to the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia with the primary diagnosis of “acute nasopharyngitis, tonsillitis of moderate severity”. PCR revealed no markers of respiratory viruses in oropharyngeal smears, no bacterial pathogens were detected by bacteriological method. Considering a positive PCR test for herpes viruses, the

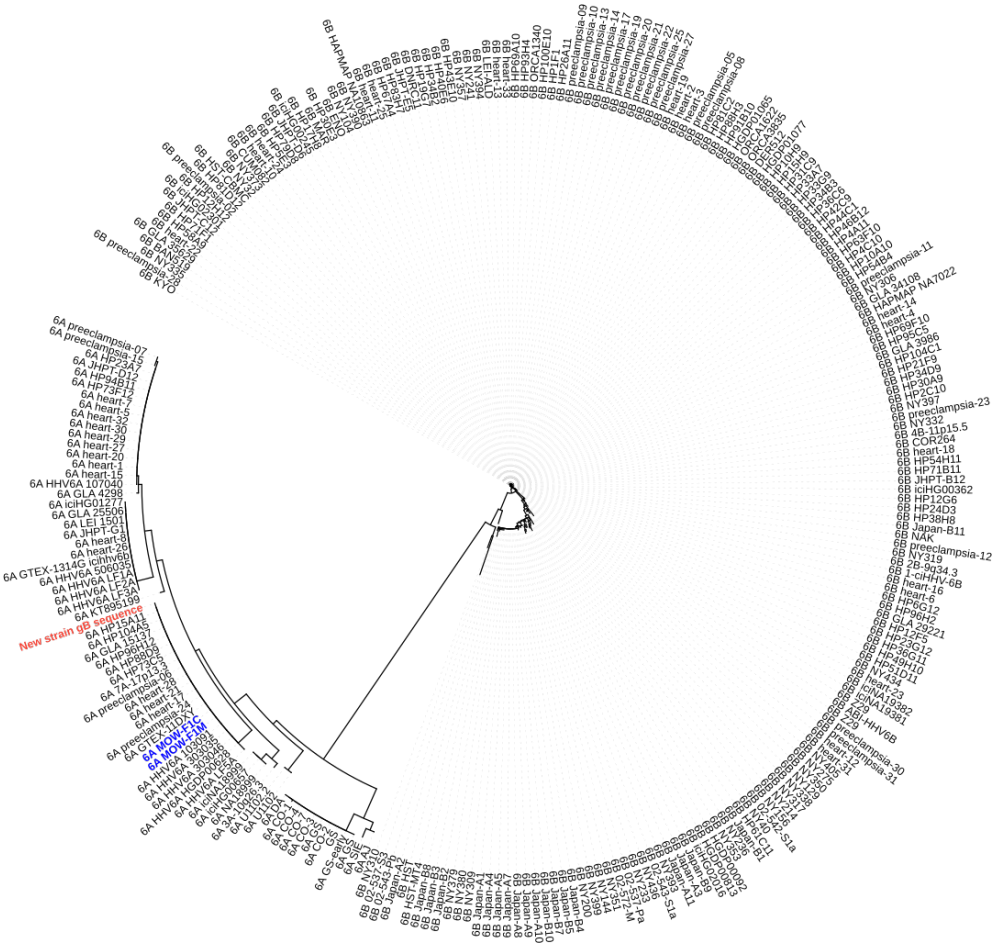

Fig. Phylogenetic position of novel type 6 herpesvirus relative to other herpes strains based on gene gB: position of new virus is highlighted in red in the tree, two viral strains from Moscow are highlighted in blue

diagnosis was clarified as "mixed etiology herpesvirus infection (HHV-6 + EBV), acute rhino-tonsillopharyngitis of moderate severity". HHV-6A/B and EBV DNA was found in peripheral blood of both patients. Positive tests for EBV DNA in blood cells, along with late IgG against EBV capsid antigen (VCA) in blood serum, proved virus reactivation. The data of laboratory tests provided in Table 1 were obtained during assessment in the first days after admission.

No CMV markers (DNA, IgM, IgG) were found in patients. Meanwhile, HHV-6 genotyping in blood and oropharyngeal smears confirmed the presence of HHV-6A variant in S.A. and diagnostically significant concentrations of HHV-6B in his brother S.T. However, no antibodies against HHV-6A/B were found in blood of S.T., which could be due to early stage of acute viral infection, before the start of antibody synthesis, or low concentrations of antibodies being outside the limits of the diagnostic test system sensitivity; high concentrations of IgG against HHV-6A/B (6.9 AU) were found in the 6-year-old patient, which could be indicative of longer infection duration.

Re-examination of these patients was performed during the follow-up visit on August 14, 2019 (Table 2).

When assessing both patients eight months later, no EBV DNA was found in blood. There were diagnostic concentrations of IgG against HHV-6A/B in blood, however, persistent HHV-6A viral load in blood and oropharyngeal smear was reported in the child S.A. over time, while no HHV-6B DNA was found in blood and oropharyngeal smear of the child S.T. Both children were clinically healthy at the time of re-examination. The fact of persistent HHV-6A isolation from blood and oropharyngeal smear of the followed-up 6-year-old patient could be associated with the viral genome integration into DNA of human cells, which required further confirmation.

To prove the HHV-6A chromosomal integration, we invited parents (mother, 36 years old, father, 39 years old) and the followed-up patients' elder brother (S.A., 14 years old), having no health complaints at the time of screening tests, for examination (Table 3).

Thus, it was found that the clinically asymptomatic father and elder brother of the patient were also characterized by high viral load represented by diagnostic concentrations of HHV-6A in blood and oropharyngeal smears. However, no HHV-6A or HHV-6B DNA was found in the mother's biomaterials. Since equally high levels of HHV-6A DNA were found in the samples of two elder brothers and the father, we suspected hereditary transmission of ciHHV-6A/B from the father to his children. It was decided to collect the parent's biomaterial other than blood with no leukocytes and cytoplasmic DNA, i.e., sperm as in the study [42], to answer the question concerning possible vertical transmission of ciHHV-6A due to technical impossibility of testing hair follicles or nail plates. DNA of spermatozoa was subjected to RT-PCR, separated from other ejaculate. After that DNA was extracted. Then we revealed the HHV-6A load of 106 gEq/mL, which was equivalent to virus concentrations in other biomaterials. This also confirmed chromosomal integration.

Later we tried to assembly the genome of this HHV-6A isolate. The HHV-6A genome sequencing involving acquisition of short and long reads of viral gene regions was carried out to confirm HHV-6A chromosomal integration and perform phylogenetic analysis.

Genome structure and position on a phylogenetic tree

We obtained a HHV-6A genome assembly, however, coverage of the reads did not exceed 3–4 reads per nucleotide, that is why genome assembly for certain genes was performed manually.

To determine the novel viral isolate phylogenetic position, we selected the gB gene, which was conventionally used to compare phylogenetic trees of herpesvirus [43]. For that the search for similar sequences of this gene among related genome assemblies using the BLAST local alignment tool was performed, and manual assembly was performed based on the results obtained. The sequences of 270 herpes virus genome assemblies were used to assess phylogenetic identity. According to phylogeny constructed for gene gB, the resulting virus strain turned out to be very similar to two strains presented by the Moscow group (GenBank ID: MK630134, MK630133) (Figure). The fact that these strains are integrated into human genome is their important feature. This conclusion can confirm our findings showing integration of novel reported strain into the host genome.

DISCUSSION

The first case of HHV-6A/B chromosomal integration was reported in early 1990s. After that the virus was often found in a number of human chromosomes: 1q, 6q, 9q, 10q, 11p, 17p, 18p, 19q, 22q, and Xp [19–23]. It is acknowledged that this is typical for both HHV-6A and HHV-6B, it is observed in telomeric chromosome regions. The paper [43] shows that the integrated HHV-6A remains inactive throughout human lifespan. The integrated virus can re-activate under exposure to various factors, which is more common for HHV-6B, and trigger infection. It has been shown that ciHHV-6A splits into clades characterized by certain chromosome and locus, in which the virus is integrated.

During the study we came across the case of prolonged HHV-6A DNA detection in biomaterials (venous blood and nasopharyngeal smears) of the patients, when performing testing at admission and during follow-up, after eight months, during the period of having no health complaints. However, viral load in venous blood and nasopharyngeal smear remained high (10^5–10^6 gEq/mL and 10^4 gEq/mL, respectively). We suspected HHV-6A chromosomal integration based on the findings. Subsequent clinical and laboratory assessment of other family members made it possible to revealed comparable high viral load in similar biomaterials of the patient's elder brother and father. Furthermore, HHV-6A was detected in the father's germ cells. Thus, it was hypothesized that the virus could be not only integrated into chromosome, but also passed to the followed-up child paternally.

We performed phylogenetic analysis based on the sequence of gene gB encoding one of the viral envelope glycoproteins to clarify the origin of HHV-6A detected in the father's germ cells. It was found that the studied HHV-6A was closely related to two assembled sequences of ciHHV-6A isolated by the research team [37] in Moscow in 2017 (GenBank ID: MK630134, MK630133). The findings confirmed the relationship of the virus we had studied with other ciHHV-6A included in the GenBank database.

CONCLUSIONS

Determination of exact position of ciHHV-6A in the chromosome locus by FISH aimed at excluding probable somatic disorders caused by chromosome structure impairment after the virus integration over time and determining the pattern of integration depending on the geographic locations of the cases revealed is an important direction of further research. Further studies will also allow us to accept or reject the earlier hypothesis that the viral genome sequence corresponds to the site of integration into human chromosome. This will make it possible to avoid using the expensive and time-consuming FISH method and adapt the tests for clinical practice.

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CLINICAL AND LABORATORY PREDICTORS OF SEVERE COMMUNITY-ACQUIRED PNEUMONIA IN CHILDREN UNDER FOUR YEARS OF AGE

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Community-acquired pneumonia (CAP) is a major cause of pediatric morbidity and mortality. Currently, there is no common approach to determination of CAP severity in children, which hampers early diagnosis and treatment of the disease. The study was aimed to determine clinical and laboratory predictors of severe CAP in children under 4 years of age. Analysis of clinical data, parameters of complete blood count (CBC), C-reactive protein (CRP) using nonparametric methods for hypothesis testing, univariate correlation analysis, cross-tabulation (Statistica 10.0), logistic regression, and ROC analysis (SPSS Statistics 20.0) was performed in 72 children aged 1 month to 3 years 11 months admitted to hospital due to CAP. Severe CAP was diagnosed in 16.7% of children. Causes of severe CAP included respiratory distress (moderate — 58.3%, severe — 16.7% of cases) and sepsis (25%). We identified significant clinical predictors of severe CAP: vomiting (OR 4.2), tachypnea (OR 28.3), chest wall retractions (OR 6), wheezing (OR 4), and the absence of rhinitis (OR 0.21). Isolated assessment of the CBC and CRP did not allow to predict CAP severity. We have developed a prediction model predicting severe CAP in children under 4 years of age based on the presence of rhinitis, tachypnea, as well as leukocyte count (sensitivity and specificity 91.7%). Thus, currently the main cause of severe CAP in children under 4 years of age is respiratory distress, in which wheezing predominates. Physical examination with an emphasis on detection of rhinitis and respiratory distress is essential for diagnosing severe CAP. The use of a pneumonia severity prediction model may contribute to improvement of management of CAP in patients under 4 years of age.

Keywords: community-acquired pneumonia, children, severity assessment, prognosis, predictor

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КЛИНИКО-ЛАБОРАТОРНЫЕ ПРЕДИКТОРЫ ТЯЖЕЛОЙ ВНЕБОЛЬНИЧНОЙ ПНЕВМОНИИ У ДЕТЕЙ ДО ЧЕТЫРЕХ ЛЕТ

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Внебольничная пневмония (ВП) — одна из ведущих причин заболеваемости и смертности детей. В настоящее время отсутствует единый подход к определению тяжести ВП у детей, что затрудняет ее раннюю диагностику и терапию. Целью работы было определить клинико-лабораторные предикторы тяжелой ВП у детей до четырех лет. У 72 госпитализированных с ВП детей в возрасте от одного месяца до трех лет 11 месяцев проводили анализ клинических данных, показателей гемограммы, уровня С-реактивного белка с помощью непараметрических методов оценки статистических гипотез, однофакторного корреляционного анализа, кросстабуляции (Statistica 10.0), логистической регрессии и ROC-анализа (SPSS Statistics 20.0). Тяжелая ВП выявлена у 16,7% детей. Причинами тяжести были дыхательная недостаточность (ДН) II и III степени (58,3 и 16,7% случаев соответственно), сепсис (25%). Выявлены значимые клинические предикторы тяжелой ВП: наличие рвоты (отношение шансов OR — 4,2), тахипноэ (OR — 28,3), втяжение уступчивых мест грудной клетки (OR — 6), синдром бронхообструкции (БОС; OR — 4) и отсутствие ринита (OR — 0,21). Изолированная оценка показателей гемограммы и уровня С-реактивного белка не позволяла прогнозировать степень тяжести ВП. Построена модель прогнозирования тяжелой ВП у детей до четырех лет, включающая наличие ринита, тахипноэ, количество лейкоцитов (чувствительность и специфичность — 91,7%). Таким образом, на современном этапе основной причиной тяжести ВП у детей до четырех лет является ДН, в патогенезе которой преобладает БОС. Физикальное обследование с оценкой синдромов ринита и ДН остается ведущим в диагностике тяжелой ВП. Модель прогнозирования тяжелой ВП может способствовать оптимизации тактики лечения.

Ключевые слова: внебольничная пневмония, дети, оценка тяжести, прогноз, предиктор

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Community-acquired pneumonia (CAP) remains the leading infectious cause of pediatric morbidity and mortality. According to the World Health Organization (WHO), about 150 million cases of CAP in children under the age of five all over the world were reported before the pandemic of novel coronavirus infection. Severe course was reported in 7–13% of CAP cases, which results in up to 20 million hospitalizations and up to 1 million deaths annually. Children under one year are most at risk of severe pneumonia, especially in the countries of South Asia and Africa [1, 2]. According to Rospotrebnadzor, in 2019, the CAP incidence in Russia was 518.9 per 100,000 population with the highest values in children (977.5 per 100,000 population); mortality rate for CAP was 3.73 per 100,000 population, including 0.28 per 100,000 population in children [3]. To date, the long-term average annual morbidity and mortality in CAP showed no downward trend, which was due to high variability of respiratory pathogens and the increase in the share of children at risk of CAP (premature babies, children with congenital malformations, organic central nervous system disorders, etc.) [4]. The emergence of new etiopathogens has a significant effect on epidemiological parameters and clinical manifestations of CAP, including severity. Thus, in the first year of the pandemic of novel coronavirus infection (2020), the number of fatal cases increased by almost 12 times and reached 44.45 per 100,000 population [3, 5].

Currently, there is no common approach to determination of CAP severity in children. This is enabled by polymorphic clinical manifestations of the disease, significant impact of the child's body response to infection, and changes in the CAP etiological structure over time. The clearly demonstrated increase in the share of viral pneumonia in children under the age of five and the decrease in the rate of local complications (pleural empyema, lung tissue destruction) determine the need to reassess the contribution of various symptoms in the disease severity [6, 7]. Different criteria of CAP severity in children have been proposed. According to the WHO, severe CAP is diagnosed in cases of children's refusal to drink, repeated vomiting, seizure, lethargy, stridor or severe protein calorie malnutrition. British Thoracic Society (BTS) has proposed 12 criteria of pneumonia severity in children, while Pediatric Infectious Diseases Society/Infectious Diseases Society of America (PIDS/IDSA) has proposed four major and 11 minor criteria, however, their diagnostic value needs clarification [8, 9]. Thus, more than a half of children having severe CAP based on the PIDS/IDSA criteria did not require hospitalization [10]. The majority of authors believe that hypoxemia, impaired mental status, age of a baby less than 3–6 months, dyspnea, multilobar infiltrates and pleural effusion on the chest X-ray (CXR) are sensitive, but mildly specific predictors of severe CAP [9].

The diagnostic significance of laboratory biomarkers associated with severe CAP in children is poorly understood, and the data available are controversial. A number of papers convincingly show that isolated WBC elevation is a significant predictor of severe CAP in children [11, 12]. The association of leukopenia below 4×10^9 kL/L with the complicated CAP and increased mortality rate (OR 6.5; 95% CI 2.7–15.6) has been revealed [13]. It has been shown that absolute neutrophil count (ANC) can be a predictor of systemic complications of pediatric CAP, including bacteremia. Elevated C-reactive protein (CRP) levels and serum levels of procalcitonin are associated with the severe course of CAP, including the development of complications (pleural empyema, lung tissue destruction, bacteremia), in cases of typical bacterial disease etiology only [14, 15]. However, no association of CRP and serum procalcitonin levels with CAP severity, including the development of hypoxemia, dyspnea and tachycardia, has been revealed [16].

The study was aimed to determine clinical and laboratory predictors of severe CAP in children under the age of four.

METHODS

Clinical follow-up of 72 children with community-acquired pneumonia (CAP) was performed January 2021 to June 2022 at the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia and St.Olga City Children's Hospital. Inclusion criteria: patients' age between 1 months and 3 years 11 months 29 days; availability of clinical, anamnestic and objective data allowing one to suspect pneumonia; detection of infiltration on CXR; pneumonia meeting the criteria for community-acquired pneumonia (occurred outside the hospital or within 72 h after hospital admission); antibiotic therapy duration at admission not exceeding 24 h. Exclusion criteria: chronic somatic disorder (including disorders of respiratory and cardiovascular systems, diabetes mellitus, confirmed immunodeficiency, etc.); history of hospital admission during previous 14 days; positive PCR test of nasopharyngeal and oropharyngeal discharge for SARS-CoV-2. The median and interquartile range (Me (IQR)) of the children's age were 2.53 (1.71–2.99) years, the male to female ratio was 1.17/1. Me (IQR) of time until hospital admission was 3 (2–4) years. The criteria for severe pneumonia were as follows: impaired vital functions resulting in the need for the child's admission to an intensive care unit (ICU), i.e. severe progressive respiratory failure (RF), impaired consciousness, peripheral microcirculation and systemic hemodynamics determined together with the critical care physician.

Admission complaints were collected and medical history was taken (disease duration, presence and type of fever, cough, catarrhal condition of the upper respiratory tract, facts of intoxication, dyspnea, vomiting, diarrhea, abdominal and chest pain), the facts of taking antibacterial drugs at the outpatient stage, vaccination against pneumococcal, hemophilic infections and influenza were clarified. Physical examination involved evaluation of the presence and severity of fever, intoxication, RF, local changes in the lungs based on percussion and auscultation, bronchial obstructive syndrome (BOS), catarrhal condition of the upper respiratory tract (based on ENT examination), lymphoproliferative syndrome, hepatomegaly and splenomegaly. Peripheral microcirculatory status was determined based on the capillary refill time (CRT): CRT < 2 s was considered as normal range. The RF symptoms were as follows: tachypnea, dyspnea (labored breathing, nasal flaring, accessory muscles involvement in respiration, grunting breathing, retractions of the chest), cyanosis, blood oxygen levels (SpO_2) decrease to less than 96% during atmospheric respiration. Age dependent criteria for tachypnea were used in accordance with the WHO guidelines: respiratory rate ≥ 60/min in children under the age of 2 months, ≥ 50/min in children aged 2–12 months, ≥ 40/min in children over the age of 12 months [1]. BOS was diagnosed when hearing prolonged expiration with a lot of bilateral wheezing. Intoxication syndrome included a number of symptoms that were considered separately: loss of appetite, decline in activity, irritability, refusal to eat or drink, drowsiness, unusual crying, lack of eye contact, impaired consciousness [17]. When there were nausea, vomiting, diarrhea (n = 17), intestinal infection was excluded by testing feces for bacteria of the genera *Shigella*, *Salmonella*, *Campilobacter*, as well as for diarrheagenic *Escherichia*, group A rotavirus, genotype II noroviruses, astroviruses, subgroup F

Table 1. Distribution of patients' complaints with significant differences depending on CAP severity

Symptom	CAP severity				OR (95% CI)	Significance level (*p*)
	Moderate		Severe			
	n	%	n	%		
Rhinorrhea	52	86.7	7	58.3	0.21 (0.05–0.8)	0.02
Dyspnea	21	35	8	66.7	3.71 (1.01–13.8)	0.04
Vomiting	15	25	7	58.3	4.2 (1.2–15.2)	0.02
Refusal to drink	2	3.3	3	25	9.7 (1.4–66)	0.03

adenoviruses by PCR (AmpliSense OKI screen-FL reagent kit; Central Research Institute of Epidemiology of Rospotrebnadzor, Russia; FRT detection format). Pulse oximetry, chest radiography with two projections, complete blood count test and serum CRP test were performed in all children. The complete blood count test performed in the Sysmex XP-300 hematological analyzer (Sysmex; Japan) involved assessment of the following parameters: white blood count (WBC), red blood cell count, hemoglobin, platelet (PLT) count, mean platelet volume and platelet distribution width, platelet larger cell ratio, erythrocyte sedimentation rate. Blood smear microscopy was used to determine the percentage of each type of white blood cells (segmented and band neutrophils, myelocytes, metamyelocytes, eosinophils, basophils, lymphocytes (Lym), plasma cells). Absolute neutrophil count (ANC) and absolute band count (ABC) were calculated considering total WBC and WBC differential.

Serum CRP levels were determined with the Taurus automated analyzer (Instrumentation Laboratory; Italy) using reagents manufactured by Vector-Best (Russia) and BioSystems (Spain).

Statistical processing of the results was performed using the Statistica 10.0 software package (TIBCO; USA) to test quantitative data for normality (Shapiro–Wilk test), calculate Me, IQR. When describing extensive characteristics, 95% confidence interval (95% CI) was calculated by the Wilson's method. Significance of differences between groups was assessed using Mann–Whitney U test (quantitative data), Fisher's exact test or Pearson's chi-squared (χ^2) test (qualitative data). Correlations between quantitative data were assessed using the Spearman's rank correlation coefficient (*r*), correlations between nominal variables in a four-column table were assessed using a φ coefficient and calculation of odds ratio (OR), while correlations between ordinal variables in the contingency tables were assessed using Somers' D. Sensitivity (Se), specificity (Sp), negative (NPV) and positive (PPV) prognostic value represented the diagnostic test characteristics. Binary logistic regression implemented in SPSS Statistics v. 20.0 (IBM; USA) was used to analyze the relationship between the independent and dependent variables; direct selection of predictors based on the likelihood function, step selection criteria (inclusion — 0.05, exclusion — 0.1) with the significance level set as $p < 0.05$ were used. The threshold values of continuous characteristics were determined by ROC analysis according to the requirement of maximum total Se and Sp. The binary classifier quality was assessed based on the area under the ROC curve (AUC). All statistical tests involved the use of critical significance level set as $p \leq 0.05$ [18, 19].

RESULTS

The condition of 12 children (16.7%; 95% CI: 9.8–26.9%) at admission was considered to be severe. In 9 children out of 12 (75%), the disease severity was determined by respiratory failure: stage II RF — 7 patients (58.3%), stage III RF — 2 patients (16.7%). Three patients out of 12 (25%) were admitted to the ICU with severe CAP due to complications: sepsis (*n* = 3; 25%) and pleural empyema (n = 1; 8.3%).

In cases of severe CAP, patients were significantly younger (Me (IQR) = 1.66 (0.96–2.59) years) compared to the cases of moderate CAP (Me (IQR) = 2.6 (2.02–3.11) years); $p = 0.008$. The logistic regression analysis showed that the likelihood of severe CAP decreased 2.6 times with increasing age factor per unit ($p = 0.009$; OR 0.39, 95% CI: 0.19–0.78).

Assessment of the patients' complaints at admission revealed significant differences for some of them depending on the pneumonia severity (Table 1).

Gender-related characteristics, features of antenatal period, duration of breastfeeding, indicators of children's physical development (at birth and at admission), as well as vaccination status against pneumococcal, hemophilic infections and influenza did not affect the risk of severe CAP ($p > 0.2$). There was also no correlation between CAP severity and body temperature increase, duration of fever, facts of intoxication and cough.

Physical examination revealed significant differences depending on pneumonia severity for some symptoms (Table 2).

We found a significant, direct, relatively strong correlation between the RF stage and CAP severity in children (Somers' D 0.68; $p < 0.001$). The relationship between BOS and various

Table 2. Distribution of physical findings with significant differences depending on CAP severity

Symptom	CAP severity				OR (95% CI)	Significance level (*p*)
	moderate		severe			
	n	%	*n*	%		
RF of any kind	29	48.3	11	91.7	11.8 (1.4–96.8)	0.005
Tachypnea	9	15	10	83.3	28.3 (5.3–151.3)	<0.001
Retraction of the chest	20	33.3	9	75	6 (1.5–24.6)	0.007
SpO_2 < 96%	18	30	8	66.7	4.7 (1.2–17.5)	0.02
Acrocyanosis	0	0	2	16.7	–	0.02
Local medium bubbling rales	18	30	0	0	–	0.03
Diffuse bilateral wheezes (BOS)	14	33.3	8	66.7	4 (1.07–14.9)	0.03

Table 3. Significant differences in hemogram parameters of children depending on CAP severity

Laboratory parameter (units)	CAP severity		OR (95% CI)*	Significance level (*p*)
	moderate Me (IQR)	severe Me (IQR)		
WBC (*10^9/L)	10 (7.6–15.1)	14.5 (11.2–22.9)	1.08 (1.004–1.17)	0.01
ANC (*10^9/L)	5 (3.1–7.6)	9.9 (4.6–15.1)	1.12 (1.01–1.24)	0.02
ABC (*10^9/L)	0.24 (0.08–0.94)	0.9 (0.3–2.5)	1.4 (1.01–2.1)	0.01
Lym (%)	31.5 (20–44.5)	19 (7–36)	0.94 (0.9–0.99)	0.02
PLT (*10^9/L)	280 (223–335)	428 (270.5–549)	1.009 (1.003-1.015)	0.02

Note: *when the laboratory parameter value increases by one.

stages of RF depending on CAP severity was analyzed. It was found that the contribution of BOS to RF was significantly larger in individuals with severe CAP (8 cases out of 11; 72.7%) compared to the cohort with moderate CAP (14 cases out of 29; 48.3%), $p = 0.03$. BOS was significantly associated with the RF stage (Somers' D 0.49; $p < 0.001$), and the correlation strength was significantly higher in the cohort with severe CAP (Somers' D 0.53; $p = 0.005$) compared to individuals with moderate CAP (Somers' D 0.25; $p = 0.03$). Laboratory parameters, for which significant differences have been revealed depending on CAP severity, are provided in Table 3.

When performing ROC analysis, cut-off points were determined for these laboratory parameters, enabling optimal differentiation between severe and moderate CAP (Table 4).

The logistic regression analysis, in which CAP severity was a dependent variable, while the listed above clinical and hematological parameters showing significant differences depending on the disease severity were independent variables, was performed to estimate rationality of the integrated assessment of clinical and laboratory parameters for diagnosis of severe CAP. We have constructed a significant ($p < 0.001$) regression model for prediction of severe CAP in children under the age of four:

$$y = \frac{1}{11+e^{(4.86+2.69*X1-4.99*X2-0.17*X3)}},$$

where y is the likelihood of severe CAP; X1 is rhinorrhea (no — 0, yes — 1); X2 is tachypnea (no — 0, yes — 1); X3 is WBC (×10^9/L). Table 5 provides characteristics of the regression model independent variables.

We determined the best cut-off probability value, $y \geq 0.305$, by ROC analysis: in case of satisfying inequality, severe CAP is predicted with Se 91.7%, Sp 91.7%, PPV 68.9%, NPV 98.2% (AUC 0.947; 95% CI: 0.889–1). When $y < 0.305$, moderate CAP is predicted with Se 91.7%, Sp 91.7%, PPV 98.2%, NPV 68.9%. In the third phase of construction the prognostic model has the following statistical characteristics: –2Log likelihood = 30.2 ($p < 0.001$), Nagelkerke's R squared coefficient 0.64 ($p < 0.001$), Hosmer–Lemeshow goodness-of-fit test 0.82 ($p = 0.66$). The lack of multicollinearity between predictors ($|r|_{max.} = 0.5$) and the distribution of resudials close to normal (Shapiro–Wilk test 0.76; $p = 0.05$) have been revealed, which suggest that the analysis conducted is correct.

DISCUSSION

The identified distribution of CAP by severity across children under the age of four is generally consistent with the literature data. The prevalence of severe CAP in the study (16.7%) is slightly higher than that in general pediatric population (7–13%) [20] and, according to other data, by at least 3% [21]. This confirms a significant impact of age factor on the likelihood of severe CAP and the maximum medical and social significance of this issue in infants and young children [22, 23]. It has been found that nowadays severity of the majority of CAPs in children under the age of four does not result from the features of early stages of ontogeny and nutrition, which can be related to improvement of the population quality of life, including reduced exposure of children to household pollutants (biofuel used for cooking, second-hand smoke, etc.) [24]. In our study, the fact of vaccination against pneumococcal, hemophilic infections and influenza had no significant effect on CAP severity in children, which was inconsistent with the available literature data [9, 24]. It can be assumed that this observation reflects alteration of CAP etiological structure in children with the increase in the share of primary viral pneumonia [6, 7].

It has been found that stage II–III respiratory failure (75%), in the structure of which bronchial obstructive syndrome significantly predominates (72.7%), is currently the main cause of severe CAP in children under the age of four. The leading role of BOS in pathogenesis of severe pneumonia is probably due to predominance of respiratory viruses in etiology of CAP in young children [6, 7]. The history of dyspnea was a weak predictor of CAP severity, which could be explained by vague understanding of the term by parents. In contrast, detection of age-depenent tachypnea (according to the WHO criteria) and retractions of the chest during physical examination significantly, many times increased the chance of severe disease (28.3 and 6 times, respectively).

Dyspepsia in the form of vomiting made a significant contribution to the development of severe CAP, increasing

Table 4. Disgnostic ability of laboratory parameters in detection of severe CAP

Laboratory parameter (units)	Cut-off point	Se (%)	Sp (%)	PPV (%)	NPV (%)	AUC (95% CI)
WBC (*10^9/L)	≥11.05	83.3	61.7	30.4	94.9	0.732 (0.6–0.86)
ANC (*10^9/L)	≥8.31	58.3	78.3	35	90.4	0.71 (0.56–0.86)
ABC (*10^9/L)	≥0.3	83.3	53.3	26.3	94.1	0.729 (0.6–0.86)
Lym (%)	≤22	66.7	71.7	32.1	91.5	0.711 (0.53–0.89)
PLT (*10^9/L)	≥423.5	58.3	90	53.9	91.5	0.714 (0.53–0.89)

Table 5. Traits included in the logistic regression model for prediction of severe CAP in children under the age of four

№ п/п	Predictors and their gradation	Code	Coefficient (B_i)	Standard error (S_i)	Wald test (W_i)	Significance level (p)	Odds ratio (95% CI)
1	Rhinorrhea: no — 0; yes — 1	X1	–2.69	1.33	4.05	0.04	0.68 (0.005–0.931)
2	Tachypnea: no — 0; yes — 1	X2	4.99	1.51	10.9	0.001	147 (7.6–2851)
3	WBC, *10^9 cells/L	X3	0.17	0.07	5.4	0.02	1.19 (1.03–1.38)
4	Constant	–	–4.86	1.97	6.1	0.01	–

the chance on average 4 times. The emergence of reflex vomiting in the structure of endogenic intoxication and faster development of exicosis in young children can constitute possible pathogenetic substantiation of this observation. The fact of vomiting is among CAP severity criteria according to BTS [8, 9], which confirms the importance of assessing this symptom in children with pneumonia.

The fact attracts attention that some symptoms earlier proposed as criteria for severe pneumonia were seldom (refusal to drink — 25%, acrocyanosis — 16.7%) or never (nasal flaring, refusal to eat, cyanosis, apnea and groaning in infants, increased CRT, impaired consciousness) reported in our study [9]. The rhinitis syndrome and local medium rales in auscultation were negative predictors of severe CAP. This interesting observation can reflect predominant involvement of upper respiratory tract and bronchi of medium caliber in individuals with mild pneumonia.

Among leukocyte indicators, absolute WBC, segmented and band neutrophil counts, relative lymphocyte counts were potential predictors of the disease severity. When assessing diagnostic value of laboratory biomarkers, the inequality $0.7 < AUC < 0.8$ was fulfilled in all cases, which was indicative of good discriminatory ability [19]. It has been found that assessment of leukocyte indicators does not improve the detection rate of severe pneumonia (positive prognostic value < 50%), but makes it possible to exclude it with high probability (negative prognostic value > 90%). It should be noted that platelet counts in individuals with severe CAP were significantly higher (1.52 times) compared to individuals with moderate CAP. Activation of the platelet component of hemostasis in severe CAP can be associated with significant involvement of the lungs being the main site of platelet formation in the disease process [25]. Other hemogram indicators, such as relative counts of immature neutrophils (band neutrophils, meta- and myelocytes) and CRP concentration were not predictors of severe pneumonia.

A significant model for prediction of severe CAP in children under the age of four was constructed using binary logistic regression. This made it possible to substantiate the feasibility of integrated assessment of clinical and hematological characteristics during examination of children with CAP aimed at early diagnosis of severe pneumonia and optimization of treatment tactics. Statistical analysis showed good quality of model approximation to hypothetic real situation, no significant differences between the reported and predicted values of the response factor and its high share of dispersion explained by the model. The model advantages include accessibility and simplicity of assessment of the proposed combination of parameters, enabling early and effective prediction of CAP severity in children under the age of four.

CONCLUSIONS

The goal of the study was achieved: clinical and laboratory predictors of severe CAP in children under the age of four were identified and assessed. Currently, respiratory failure, in the pathogenesis of which BOS predominates, is the main cause of severe pneumonia. Clinical assessment of patient's condition focused on detection of the rhinitis syndrome and RF, including age-dependent tachypnea and retraction of the chest, plays a leading role in the diagnosis of pediatric CAP. Isolated assessment of hematological parameters and serum CRP levels makes it impossible to predict pneumonia severity. A model for early prediction of CAP severity in children under the age of four has been proposed, the use of which can contribute to the treatment tactics improvement. Given small size of the sample used in the study (72 patients) and no consensus about the criteria of severe CAP diagnosis based on the literature data, further research with the prospect of creating a validated quantitative system for assessment of pneumonia severity in children is necessary.

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ISOLATION AND CHARACTERIZATION OF VIRULENT BACTERIOPHAGES AGAINST *KLEBSIELLA PNEUMONIAE* OF SIGNIFICANT CAPSULAR TYPES

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The growing proportion of antibiotic-resistant *Klebsiella pneumoniae* strains raises challenges to the healthcare system and requires the development of alternative treatment options. Bacteriophage therapy is one of such options. The study was aimed to isolate and describe bacteriophages effective against *K. pneumoniae* strains of clinically significant capsular types. The bacteriophages were isolated from the sewage and river water samples using the enrichment culture technique. The spectrum of lytic activity of the phages was tested on the collection of *K. pneumoniae* clinical isolates (n = 279). The studied bacteriophages lysed 52.8–100% of *K. pneumoniae* strains of respective capsular types: phage VKV295 lysed 100% of strains with the capsular type KL1, SAA231 — 52.8 of strains with KL2, NNK-G4 — 100% of strains with KL39, VSG32 — 66.7% of strains with KL41, NKA196 — 87.5% of strains with KL47, Rappa3 — 87.5% of strains with KL57, PEA128 — 95.5% of strains with KL64, and ChM-G5 — 69.6% of strains with KL102. Whole-genome sequencing and subsequent bioinformatic analysis revealed that the phages belong to the *Autographiviridae* family and are classified into three genera.The lytic spectrum of phages was limited to specific capsular types due to the presence of specific receptor-binding proteins, polysaccharide depolymerases. The isolated bacteriophages were strictly virulent, did not carry harmful genetic determinants, and had a specific host range, making them applicable in therapeutic practice for combating antibiotic-resistant infections caused by *K. pneumoniae*.

Keywords: virulent bacteriophages, *Klebsiella pneumoniae*, antibiotic resistance, polysaccharide depolymerases

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Author contribution: Gorodnichev RB — study plan, data acquisition and processing, manuscript writing; Kornienko MA — study plan, data acquisition and processing; Bespiatykh DA — data processing; Malakhova MV, Krivulia AO — data acquisition; Veselovsky VA, Goloshchapov OV, Chernenkaya TV, Bespyatykh YuA — data acquisition and processing; Shitikov EA — study plan, o data processing, manuscript writing.

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ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА ВИРУЛЕНТНЫХ БАКТЕРИОФАГОВ ПРОТИВ *KLEBSIELLA PNEUMONIAE* ЗНАЧИМЫХ КАПСУЛЬНЫХ ТИПОВ

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В контексте растущей устойчивости к антибиотикам бактериофаги — альтернатива традиционной антимикробной терапии. Терапия бактериофагами — одна из таких альтернатив. Целью исследования были выделение и характеристика бактериофагов, эффективных против штаммов *Klebsiella pneumoniae* клинически значимых капсульных типов. Из проб сточных и речных вод методом накопительных культур было выделено восемь фагов. Определение спектра литической активности фагов проводили на коллекции клинических изолятов *K. pneumoniae* (n = 279). Бактериофаги лизировали 52,8–100% изолятов *K. pneumoniae* соответствующих капсульных типов: фаг VKV295 — 100% изолятов с капсульным типом KL1, SAA231 — 52,8% с KL2, NNK-G4 — 100% с KL39, VSG32 — 66,7% с KL41, NKA196 — 87,5% с KL47, Rappa3 — 87,5% с KL57, PEA128 — 95,5% с KL64 и ChM-G5 — 69,6% с KL102. Их геномы были секвенированы и проанализированы биоинформатически. Фаги принадлежали к семейству *Autographiviridae* и относились к трем родам. Литический спектр фагов был ограничен конкретными капсульными типами вследствие наличия специфичных рецептор-связывающих белков — полисахаридеполимераз. Выделенные бактериофаги были строго вирулентными, не несли вредных генетических детерминант, что позволяет их применять в терапевтической практике для борьбы с антибиотикорезистентными инфекциями, вызванными *K. pneumoniae*.

Ключевые слова: вирулентные бактериофаги, *Klebsiella pneumoniae*, антибиотикорезистентность, полисахарид-деполимеразы

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Klebsiella pneumoniae is a Gram-negative, rod-shaped bacterium belonging to the *Enterobacteriaceae* family. Bacteria of this species are the cause of many human infectious diseases. Pneumonia (inflammation of the lungs) is the best known one, however *K. pneumoniae* can also cause urinary tract infections, bloodstream infections, wound infections, and sepsis [1]. Antibiotic therapy remains the main method to prevent and treat infections caused by *K. pneumoniae*, despite the fact that the share of mulridrug-resistant strains can reach 20–30% [2, 3]. The *K. pneumoniae* infection mortality is as high as 38%, while the annual number of deaths associated with antibiotic resistance is 650,000 people [4, 5].

Bacteriophage therapy is considered to be a simple, safe and highly effective alternative to antibiotics [6]. Bacteriophages are the largest and most common group of viruses; they have been used as antimicrobials since their discovery in the early 20th century. Today, monophages and cocktails of several lytic phages are successfully used for personalized therapy [7–9]. However, commercially available broad-spectrum phage cocktails have limited efficacy [10].

The *K. pneumoniae* bacteriophage efficacy is largely defined by the type of capsular polysaccharide of the host bacterium [11]. The *K. pneumoniae* polysaccharide capsule is a key factor of virulence protecting the bacterium against environmental factors, including host immunity [12]. Today, more than 100 different polysaccharide capsule types are distinguished based on the conventional serological method and the method of sequencing distinct genes of the cps gene cluster, some of them (KL1, KL2, KL8, KL20, KL39, KL41, KL47, KL53, KL57, KL64, KL102 и KL107) are associated with increased virulence and antibiotic resistance [13–17].

The *K. pneumoniae* bacteriophages are adsorbed on the surface of bacteria, they dissolve the polysaccharide capsule with the specialized enzymes, polysaccharide depolymerases, usually found on the phage tail fibers fiberand spikes. Polysaccharide depolymerases possess enzyme activity against certain bond between monosaccharides in the polysaccharide monomer [11].

The study was aimed to isolate and describe bacteriophages capable of lysing *K. pneumoniae* strains of clinically significant capsular types.

METHODS

Bacterial strains and their characteristics

The collection of (n = 279) *K. pneumoniae* clinical isolates was compiled in 2018–2022: 79 strains were obtained from the Raisa Gorbacheva Memorial Research Institute for Pediatric Oncology, Hematology and Transplantation (Saint Petersburg, Russia), 66 from the Sklifosovsky Research Institute for Emergency Medicine (Moscow, Russia), 64 from the collection of the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia (Saint Petersburg, Russia), 58 from the Clinical Hospital № 123 of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia (Odintsovo, Russia); 12 isolates were generously provided by SCPM-Obolensk (Obolensk, Russia).

Bacterial strains were grown using the lysogeny broth (LB) (Himedia; India) at 37°C. Bacterial species were identified by MALDI-TOF mass spectrometry [18]. The fact of the *K. pneumoniae* belonging to certain capsular type was determined by the *wzi* gene sequencing [19].

Bacteriphage isolation and purification

Hospital sewage, from which *K. pneumoniae* strains were isolated, and water of the Likhoborka (Moscow) and Klyazma (Korolev) rivers were used as the sources of bacteriophages.

To eliminate bacterial component, the sample of sewage or river water was centrifuged at 4000 g for 10 min, supernatant was filtered using the 0.22 μm filters (Merk Millipore; USA). Equal amounts (15 mL) of filtered water and double concentration LB broth were combined and inoculated with 20 μL of the overnight culture of the potential bacterial host strain. This mixture was incubated overnight on the shaker at 37 °C. The resulting suspension was sterilized by filtering through the 0.22 μm filter, and the presence of bacteriophages in the filtered liquid was confirmed by the spot test [20]. Isolation and buildup of pure bacteriophage culture was accomplished via three passages through a single plaque.

The study also involved NER40 bacteriophage isolated from the Chermyanka river (Moscow) that was described in the previous paper [21].

Determination of lytic spectrum

The lytic spectra of bacteriophages were defined by the spot test assay [20]. For that 100 μL f the culture of each *K. pneumoniae* strain grown to logarithmic phase (OD_{600} = 0.3) were mixed with 5 mL of semi-solid agar in LB (0.7% agar) and distributed among the Petri dishes with thin layer of agar in LB (1.5% agar). Testing included application of 5 μL of monophage lysates with a titre of 106 PFU/mL to the surface of fresh lawns of the tested *K. pneumonia* strains. Then the Petri dishes were incubated at 37 °C overnight. Lytic activity of bacteriophages was determined based on the presence of the zone of continuous bacterial cell lysis matching the shape of initial drop. The presence of translucent area surrounding the zone of lysis was interpreted as polysaccharide depolymerase activity.

Whole-genome bacteriophage sequencing and bioinformatics data analysis

The phage genomic DNA was extracted using the standard phenol–chloroform extraction protocol [22]. Sequencing was carried out using the MiSeq tool (Illumina; USA) and the MiSeq Reagent Nano Kit v2 (500 cycle) (Illumina; USA) in accordance with the manufacturer's instructions. Genomes were assembled with the SPAdes software (v. 3.14.0). The GeneMarkS online service (v. 4.32) was used to identify open reading frames (ORFs) in the genome. Assessment of tRNA genes was performed with ARAGORN (v. 1.2.41).

Genes were predicted and annotated manually using BLASTp, HHPred, and InterPro. To confirm the lack of genes encoding toxins and antibiotic resistance determinants, comparison with the databases containing virulence factors of pathogenic bacteria [23] and antibiotic resistance genes [24] was performed. The annotated sequences of bacteriophage genomes were deposited in the GenBank database.

Phylogenetic analysis involved 40 reference bacteriophage genomes proposed by the International Committee on Taxonomy of Viruses (ICTV). Phylogenetic trees were constructed based on the bacteriophage complete genomes using the VICTOR tools [25]. The closest homologues among bacteriophages were determined with the BLASTn algorithm. Comparative analysis of distinct protein sequences was accomplished using the BLASTp service. Comparative analysis of complete genomes was performed using the Circoletto tools [26].

RESULTS

Characteristics of *K. pneumoniae* strains

The *wzi* gene nucleotide sequence was determined for all 279 strains of the collection. Comparative analysis of the

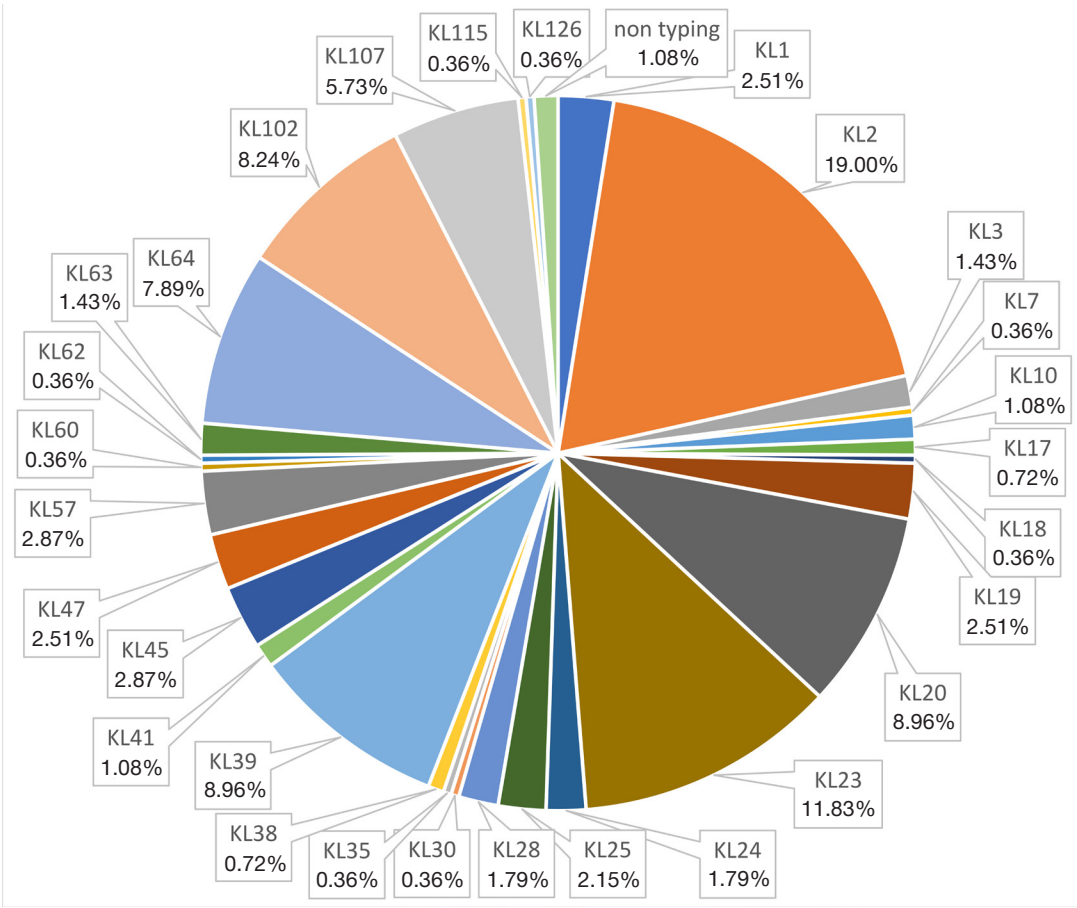

Fig. 1. Diversity of capsular types in the *K. pneumoniae* collection

resulting sequences and the sequences from the Institut Pasteur database made it possible to determine the alleles corresponding to distinct capsular types. A total of 40 unique *wzi* gene allele variants were found, among which 37 were associated with certain capsular types; no associations with the known capsular types were found for three variants (*wzi* 475, *wzi* 493 and *wzi* 163). The collection included 29 different capsular types, among which seven constituted 70% of all isolates: KL2 (19%), KL23 (12%), KL20 (9%), KL39 (9%), KL64 (8%), KL102 (8%), and KL107 (6%) (Fig. 1). The shares of other capsular types, often associated with high virulence, were less than 5%: KL1 — 3%, KL41 — 1%, KL47 — 3%, KL57 — 3%.

Isolation, phenotypic characteristics and lytic spectrum of bacteriophages

A total of eight bacteriophages (VKV295, SAA231, NKA196, NNA-G4, VSG32, Rappa3, PEA128, and ChM-G5) lysing the *K. pneumoniae* strains of eight clinically significant capsular types (KL1, KL2, KL39, KL41, KL47, KL57, KL64, and KL102) were extracted from three sewage samples and two river water samples. Strains of these capsular types constituted 53.05% of the collection.

The majority of bacteriophages formed small, round, transparent plaques (1–2 mm) surrounded by the 1–2 mm halo. Certain bacteriophages (VKV295 and Rappa3) formed larger round, transparent plaques (2–4 mm) also surrounded by halo (Fig. 2, Table 1).

Bacteriophages showed high specificity of the lytic spectrum: each isolated phage was capable of lysing only strains with the same capsular type as the strain, on which the bacteriophage was isolated. All the studied bacteriophages lysed 52.8–100% of strains of certain capsular types (Table 1). The previously described bacteriophage NER40 specifically lysing strains with the capsular type KL2 was included in the study for reference [21].

Whole-genome bacteriophage sequencing and phylogenetic analysis

Complete genomes of phages were assembled and deposited in the NCBI GenBank database (Table 2). The genome size varied between 39058 and 44575 bp, the G + C content was 50.4–54.3%. All phage genomes had terminal repeats sized 167–282 bp on both ends. No tRNA genes were found in the phage genomes, and the number of open reading frames (ORFs) predicted for various bacteriophages was 42–53 (Table 2).

Phylogenetic analysis has shown that all the studied bacteriophages belong to three genera of the family *Autographiviridae* (Fig. 3). Phages VKV295, SAA231, NKA196, and NNA-G4 belong to the genus *Drulisvirus*, Rappa3 and PEA128 are members of the genus *Przondovirus*, while VSG32 and ChM-G5 belong to the genus *Teetrevirus*. According to the BLASTn analysis results, the closest homologues of *Drulisvirus* phages were represented by KpV2883 (GenBank MT682065.1; 90.53% identity) for phage VKV295, vB_KpnP_KpV74 (GenBank NC_047811.1; 88.12% identity) for phage SAA231, and KPPK108.1 (GenBank OK583892.1; 90.56% and 85.03% identity) for NNA-G4 and NKA196. The closest homologues of phages Rappa3 and PEA128 were represented by phages of the genus *Przondovirus* K5-2 (GenBank NC_047798.1; 81.32% identity) and 066037 (GenBank MW042800.1; 86.27% identity), respectively. Homologues of phages *Teetrevirus*VSG32 and ChM-G5 were represented by *Salmonellaphage* phiSG-JL2

Table 1. Microbiological characteristics of bacteriophages

Source of bacteriophages	Bacteriophage	Bacteriophage capsular specificity	Number of lysed strains of certain capsular type	Plaque morphology	Halo, mm
				Plaque, mm	
Sewage of the Clinical Hospital No. 123 of the Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of FMBA of Russia	VKV295	KL1	7/7	2	3
	NNA-G4	KL39	25/25	0,5–1	1
Sewage of the Sklifosovsky Research Institute for Emergency Medicine	Rappa3	KL57	7/8	4	3
	VSG32	KL41	2/3	1–2	1–3
Sewage of the Raisa Gorbacheva Memorial Research Institute for Pediatric Oncology, Hematology and Transplantation	PEA128	KL64	21/22	1–2	1
	ChM-G5	KL102	16/23	1	1
Klyazma river	SAA231	KL2	28/53	1	1
Likhoborka river	NKA196	KL47	7/8	1–2	2–3
Chermyanka river	NER40 [21]	KL2	49/53	3–5	2–4

(GenBank NC_010807.1; 84.00% identity) and *Klebsiellaphage* 6998 (GenBank OL362282.1; 90.13 % identity), respectively (Table 2).

Functional annotation and comparative analysis of genomes

All the studied bacteriophages were members of the family *Autographiviridae* and, therefore, had similar genome structure: all genes were located on the leading DNA strand, phages encoded both DNA and RNA polymerases, while genes of nucleic acid metabolism and genes encoding structural proteins formed clusters in the left and right parts of the genome, respectively. Members of this family are virulent phages that carry no integrase genes. The annotated genes of the studied bacteriophages include no genes encoding integrases, antibiotic resistance determinants, toxins or any other known genes that are potentially unfavorable in terms of therapy.

The genomes of phages of the genus *Drulisvirus* carried 51–53 ORFs, among which 22–24 were annotated as genes encoding hypothetical proteins, 12–14 were nucleic acid metabolism genes, 12–13 were genes encoding capsid proteins; there were also three genes responsible for host cell lysis represented by the genes encoding spanin, choline and endolysin following one another.

Each of four phages of the genus *Drulisvirus* carried two genes encoding phage fiber proteins, however, both genes encoded polysaccharide depolymerase domains only in VKV295; in three other phages, a depolymerase domain was found on one fiber out of two only. The fiber genes of phage VKV295 (*orf0043* and *orf0051*) carried glycoside hydrolase family 28 and K1 lyase domains and showed 82.53 and 99.75% identity with the fibers of phage KpV2883 that was considered to be the closest based on BLASTn. In turn, the fiber genes of bacteriophage SAA231 showed 96.18 and 97.57% identity with the closest homologue, phage vB_KpnP_KpV74; the first fiber gene (*orf0044*) carried no depolymerase domain, while the second one (*orf0052*) encoded the glycoside hydrolase family 28 domain. This depolymerase (SAA231_ orf0052) showed 98.1% homology with the earlier reported fiber *orf0053* of phage NER40. Bacteriophage NNA-G4 carried two fiber genes, among which only one (*orf0052*) encoded depolymerase with pectate lyase 3 domain and showed 95.65% identity with the fiber gene of phage VLC5 (GenBank MT197175.1; 74.97% identity). As in NNA-G4, only the second fiber of phage NKA196 (*orf0052*), which showed 99.13% identity with the fiber of phage

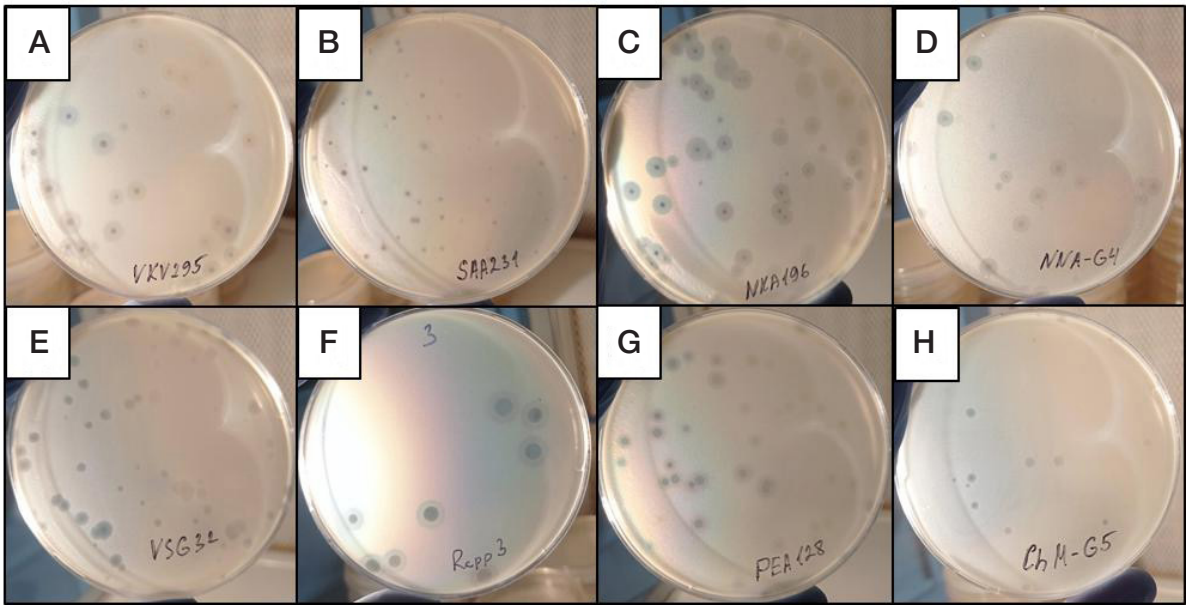

Fig. 2. Plaque morphology of phages VKV295 **(A)**, SAA231 **(B)**, NKA196 **(C)**, NNK-G4 **(D)**, VSG32 **(E)**, Rappa3 **(F)**, PEA128 **(G)**, and ChM-G5 **(H)**

Table 2. Genetic characteristics of bacteriophages

Bacteriophage	GenBank	Taxonomic status	Size, bp	ORF	G + C	Identity with the closest homologue, %
VKV295	OR287807	*Drulisvirus*	42380	51	54.10%	90.53
SAA231	OR287809	*Drulisvirus*	44281	53	54.30%	88.12
NNA-G4	OR287810	*Drulisvirus*	44575	52	53.80%	90.56
NKA196	OR287808	*Drulisvirus*	44083	52	53.90%	85.03
Rappa3	OR287806	*Przondovirus*	40593	42	53.10%	81.32
PEA128	OR287812	*Przondovirus*	40386	47	52.80%	86.27
VSG32	OR287811	*Teetrevirus*	39058	48	50.40%	84
ChM-G5	OR287804	*Teetrevirus*	39235	45	50.90%	90.13

KPPK108.2 (GenBank OK583892.1; 85.03 % identity), carried a depolymerase domain of glycoside hydrolase family 28.

Genus *Przondovirus* was represented by two phages, the genomes of which carried 42–47 ORFs. As a result of the annotation, we managed to predict the functions of 71.2–73.8% of hypothetical proteins. A total of 15–16 nucleic acid metabolism genes, 14–15 structural genes, and two genes responsible for host bacterium lysis represented by class II choline and Rz-like spanin were annotated.

Rappa3 bacteriophage had two fibers (*orf0037* and *orf0038*) containing depolymerase domains represented by pectate lyases 3. The first fiber showed 29.28% identity with the fiber of phage K11 (GenBank NC_011043.1; 81.01% identity), while the second one showed 71.38% identity with the fiber of phage vB_KpnP_KpV767 (GenBank NC_047772.1; 78.09% identity). A single fiber of phage PEA128 showed 99.72% identity with the fiber of phage TUN1 (GenBank HG994092.1; 84.11% identity) and carried the glycoside hydrolase family 28 domain.

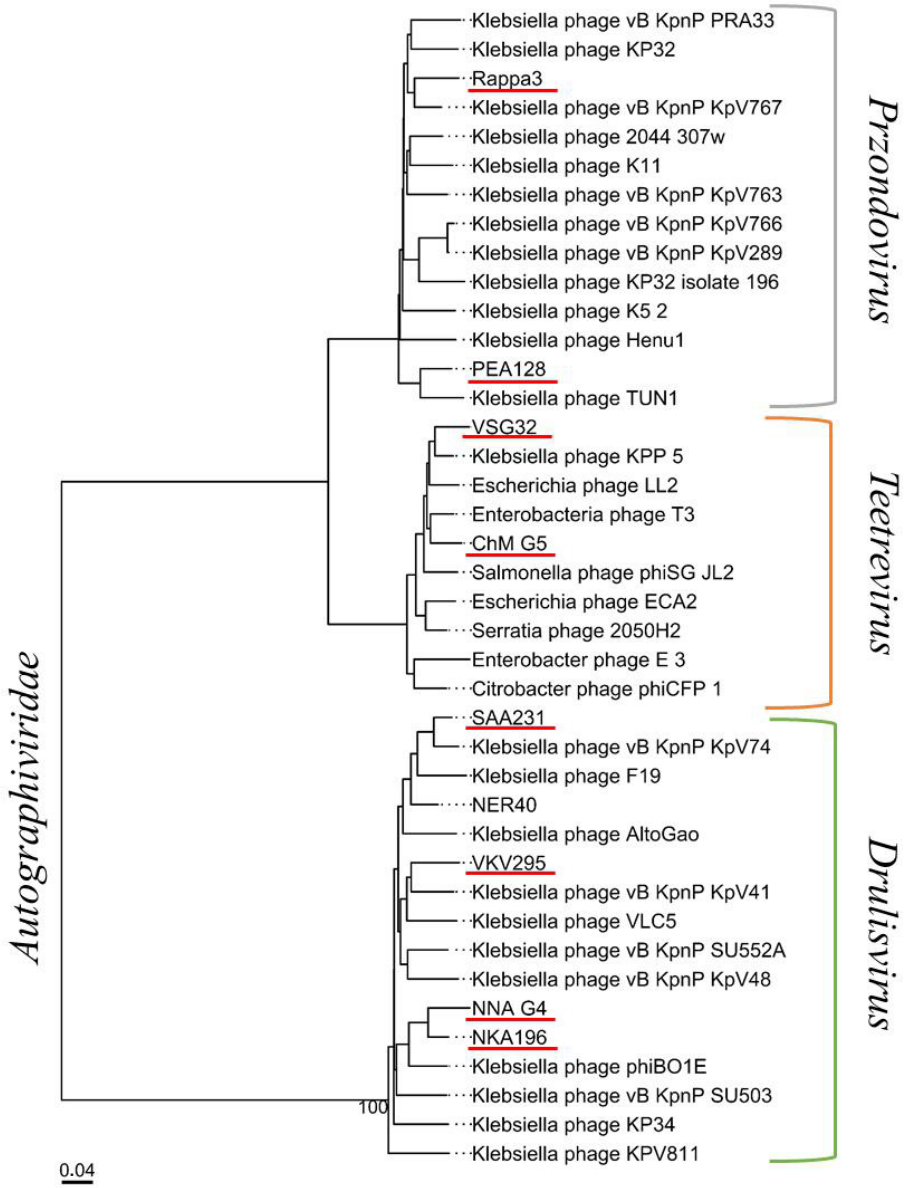

Fig. 3. Phylogeny of the *K. pneumonia* bacteriophages. The studied bacteriophages are highlighted in *red*

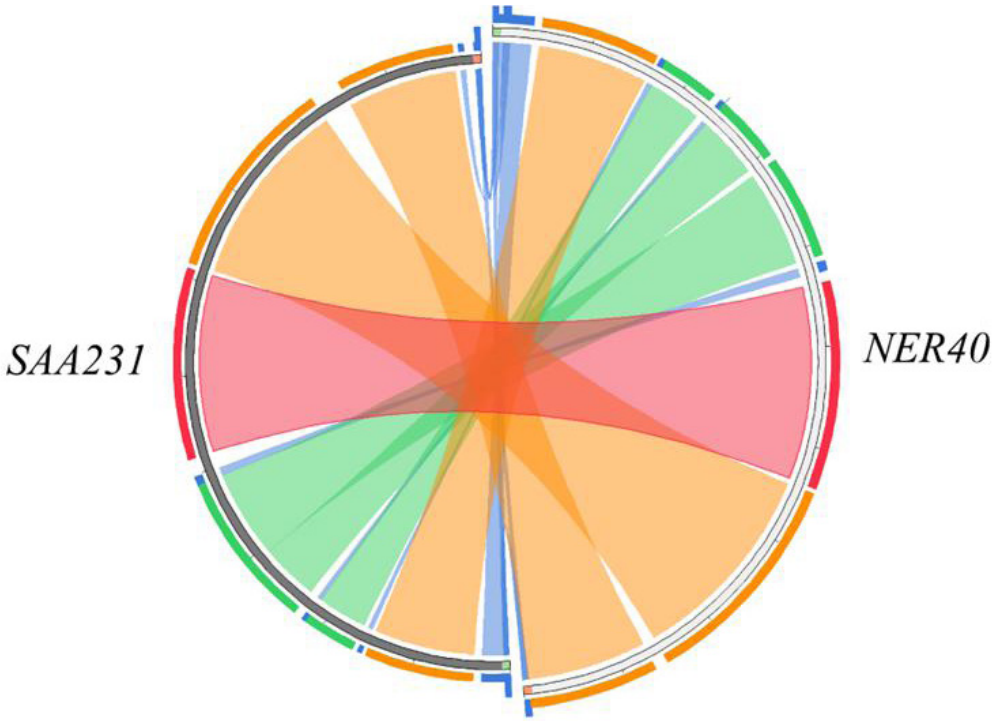

Fig. 4. Comparison of the NER40 and SAA231 phage genomes (percentage of homology is highlighted in *blue* < 25%; *green* < 50%; *orange* < 75%; *red* >75%)

Bacteriophages VSG32 and ChM-G5 of the genus *Teetrevirus* had 48 and 46 ORFs, respectively, among which functions were predicted for 83.33 and 78.26% ORFs: 21 and 19 ORFs encoded nucleic acid metabolism genes, 17 and 15 encoded structural genes. Genes responsible for host lysis were organized in the same manner as in phages of the genus *Przondovirus* and represented by two ORFs encoding choline and Rz-like spanin.

Each of the isolated phages carried one fiber gene encoding the receptor-binding protein. Depolymerase of phage ChM-G5 was represented by the *orf0040* fiber showing 90.41% identity with the fiber of phage 6998 (GenBank OL362282.1; 90.13% identity) and carrying the pectate lyase 3 domain. BacteriophageVSG32 encoded the *orf0042* fiber showing 94.97% identity with the fiber of phage KPP-5 (GenBank MW600722.1; 87.70% identity) and carrying an adhesion domain of indeterminate nature.

DISCUSSION

The *K. pneumoniae* strains taken as host strains have the capsular types associated with nosocomial infections that are difficult to treat due to the presence of antibiotic resistance determinants [3, 16, 27]. These strains are widespread in Russia and neighboring countries, they often carry genes responsible for carbapenem and broad spectrum β-lactam antibiotic resistance, as well as genes responsible for hypervirulence [16, 27]. Strains with the capsular types KL1, KL2, KL39, KL41, KL47, KL57, KL64, and KL102 constitute 53.05% of the collection compiled, which means high relevance of isolating therapeutic bacteriophages against them.

Novel bacteriophages were isolated from sewage of the same hospitals, where the strains of the collection were isolated, as well as from water of the rivers flowing through Moscow. All the isolated bacteriophages formed specific translucent halos surrounding individual plaques, which was a characteristic feature of the presence of receptor-binding proteins represented by polysaccharide depolymerases. This is also confirmed by the narrow range of phage hosts limited to *K. pneumoniae* strains of specific capsular types. Bacteriophages specific for *K. pneumoniae* strains of the capsular types KL1, KL2, KL47, KL57, KL64, and KL102 were earlier described in the literature as members of different taxons. However, to date, only one phage specific for *K. pneumoniae* strains of the capsular type KL39 have been reported; no phages able to specifically lyse *K. pneumoniae* strains of the capsular type KL41 have been reported [28].

The analysis of genomes of the isolated bacteriophages has shown that all phages are members of the family *Autographiviridae* and are more than 5% different from the closest phages presented in the NCBI database, which allows us to say that the isolated bacteriophages are new species of appropriate genera [29]. Despite the differences between complete genomes sufficient for identification of new species, the fiber genes responsible for phage adsorption on the surface of bacteria and largely determining the host range showed higher degree of homology with the earlier reported bacteriophage fibers. Thus, for example, fibers of phages VKV295, SAA231, NKA196, and PEA128 turned out to have 82.53–99.75% homology with the fibers of earlier characterized bacteriophages KpV2883, vB_KpnP_KpV74, KPPK108.2 and TUN1. In contrast, fibers of phages NNA-G4, ChM-G5, VSG32 and Rappa3 were either homologous to bacteriophages with undescribed host specificity, or showed poor (< 75%) homology with the closest fibers of the known phages (based in BLASTp).

Interesting is the fact that our collection includes the earlier reported bacteriophage NER40 (GenBank MZ602146.1) of the genus *Drulisvirus* specific for *K. pneumoniae* strains with the capsular type KL2 [21]. A significant difference between the two bacteriophages was that, while specifically lysing *K. pneumoniae* strains with the capsular type KL2, bacteriophage NER40 showed higher efficiency, 49/53 (90.57%) vs. 28/53 (52.8%) for SAA231. The main differences between genomes of phages NER40 and SAA231 are within the region between 6.5–17.5 kbp, where the genes responsible for life cycle are located, while the genes of adsorption apparatus have shown

high degree of homology (98.1%) (Fig. 3). Given the above, such significant differences in the host ranges can be due to the differences in the success in bypassing bacterial antiphage defense systems, such as restriction modification system and CRISPR. It can be assumed that the genes ensuring successful bypassing of such systems are located in this specific region of the phage genome (6.5–17.5 kbp) and determine the differences in potential therapeutic efficacy.

It is important to note that no potentially undesired determinants have been found in the genomes of isolated bacteriophages, which, along with their phylogenetic position, characterizes them as strictly virulent bacteriophages suitable for antibacterial therapy. In turn, high lytic activity of phages and the presence of polysaccharide depolymerases as receptor-binding proteins make it possible to use both bacteriophages and their derivatives for therapy.

CONCLUSIONS

We have isolated and characterized bacteriophages possessing specific lytic activity against clinically significant *K. pneumoniae* strains of certain capsular types: VKV295 against KL1, SAA231 against KL2, NNK-G4 against KL39, VSG32 against KL41, NKA196 against KL47, Rappa3 against KL57, PEA128 against KL64, ChM-G5 against KL102. The phage genomes were tested for any genes potentially dangerous for therapy (integrases, toxins, antibiotic resistance factors), which means that these phages may be used for treatment.

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DETECTION AND PREVENTION OF IRON DEFICIENCY IN DONORS OF BLOOD (BLOOD COMPONENTS)

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The problem of iron deficiency among donors is relevant and directly affects the provision of hemocomponents to the blood service. Donors, being a risk group for the development of iron deficiency, are examined before donation, including a study of hemoglobin levels. However, there is no information about the state of iron stores, when depleted, iron deficiency anemia develops. In turn, anemia is a contraindication to donation and, therefore, leads to medical exemptions from donation. The purpose of the study was to evaluate the main indicators of iron metabolism in donors of blood and (or) blood components at risk of developing latent iron deficiency. The examination of 174 donors included a hemogram, assessment of the level of hemoglobin, serum ferritin (SF), transferrin, and soluble transferrin receptors. When assessing the intensity of changes in reserve and transport iron indicators, 228 deviations from the reference range were analyzed. The criterion for the risk of developing iron deficiency was hemoglobin values at the lower limit of normal (130–135 g/l in men and 120–125 g/l in women) and the threshold level of ferritin (30 μg/l in male donors and 20 μg/l in women). The risk group included 58.3% of young donors — women who donate blood 1–2 times during the year ($p < 0.01$) and 66.6% ($p < 0.01$) of donors — men who donate blood regularly throughout 4 and > years. The average ferritin level in male donors was 27.37 μg/l ($p < 0.02$) and lower than the reference values. It is concluded that it is advisable to assess the indicators of iron metabolism in donors in the case of borderline hemoglobin levels, in women of reproductive age after 2 blood donations and in men with the number of donations ≥ 10. To replenish the iron depot in the body, when iron deficiency is detected in donors, it is necessary to consider the issue of prevention.

Keywords: iron deficiency, donation, risk, ferritin, transport iron

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ВЫЯВЛЕНИЕ И ПРОФИЛАКТИКА ЖЕЛЕЗОДЕФИЦИТНОГО СОСТОЯНИЯ У ДОНОРОВ КРОВИ (КОМПОНЕНТОВ КРОВИ)

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Проблема дефицита железа среди доноров является актуальной и напрямую влияет на обеспечение гемокомпонентами службы крови. Доноры, являясь группой риска по развитию железодефицитного состояния, проходят обследование перед донацией, включающее исследование уровня гемоглобина. При этом отсутствует информация о состоянии запасов железа, при истощении которых развивается железодефицитная анемия. В свою очередь анемия является противопоказанием к донорству и, следовательно, приводит к медицинским отводам от донации. Целью исследования было оценить основные показатели обмена железа у доноров крови и (или) компонентов крови, подверженных риску развития латентного железодефицита. Обследование 174 доноров включало гемограмму, оценку уровня гемоглобина, сывороточного ферритина (СФ), трансферрина, растворимых рецепторов трансферрина. При оценке интенсивности изменений показателей запасного и транспортного железа были проанализированы 228 отклонений от референтного диапазона. Критерием риска развития железодефицитного состояния были значения гемоглобина у нижней границы нормы (130–135 г/л у мужчин и 120–125 г/л у женщин) и пороговый уровень ферритина (30 мкг/л у доноров-мужчин и 20 мкг/л у женщин). В группу риска вошли 58,3% молодых доноров-женщин, сдающих кровь 1–2 раза в течение года ($p < 0,01$) и 66,6%, ($p < 0,01$) доноров-мужчин, сдающих кровь регулярно в течение четырех и более лет. Средний показатель ферритина у доноров-мужчин — 27,37 мкг/л ($p < 0,02$) был ниже референсных значений. Сделан вывод о целесообразности оценки показателей обмена железа у доноров в случае пограничного уровня гемоглобина, у женщин репродуктивного возраста после 2 донации крови и мужчин с числом донаций ≥ 10. Для восполнения депо железа в организме при выявлении железодефицита у доноров необходимо рассматривать вопрос о профилактике.

Ключевые слова: железодефицит, донация, риск, ферритин, транспортное железо

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In every blood donation, iron loss can promote latent iron deficiency (LID) in recurrent donors, especially among women. Progression of iron deficiency results in iron deficiency anemia, which subsequently becomes the reason for temporary exemption of donors from donation [1–6]. Iron deficiency can be accompanied with such symptoms as weakness, absent-minded behavior, somnolence, fatigue, taste disturbances, skin dryness, severe loss of hair, fragility and deformity of nail plates, gastrointestinal disturbances, menstrual disorder in females, etc. It is known that not only whole blood collection is accompanied with iron loss. Apheresis damages red blood cells, which go back to the blood stream [7]. Thus, when platelets are donated using apheresis, donors lose up to 100 ml of blood. Then there is risk that iron deficiency can be developed. The majority of values (Hb, HCT, transferrin, transferrin saturation and ferritin) were significantly lower than the reference values [8]. With the increased interval between donations, the percentage of donors with iron deficiency dropped [9]. An increased rate of apheresis can trigger low iron [10]. It should be noted that after donation iron deficiency anemia can be developed in 0.14–0.8% of male donors only. For female donors, the value is a sequence higher. It is 1.7–17.4%. Donation of 450 ± 10% ml of whole blood results in Hb drop in a donor by 3.5–14 g/L from baseline. Each donation results in the loss of 200 to 250 mg of iron. It is about 5-6% of entire iron stores in the body [11]. Maximum Hb drop is seen at day 5 post-donation. It gets gradually replenished to the pre-donation value at an average of about 30 days. To synthesize new Hb molecules, a healthy donor uses the available iron stores. Taking into account stages of iron deficiency, WHO recommends to determine the concentration of both Hb and ferritin [11,12] in order to diagnose iron deficiency among people who look healthy. It happens because plasma/serum ferritin is positively correlated with total iron stores in the lack of inflammation [13–15]. At the stage of latent iron deficiency, lab values of serum ferritin (SF) have more pronounced changes. Not only depletion of iron depot such as low serum ferritin but also low iron concentration in serum and carrier proteins are recorded. Decrease of serum ferritin below 15 μg /l in adults (adjusted below 30 μg/l) and 70 μg /l in adults with inflammatory diseases means inevitable drop of Hb in the future [12].

By now, numerous works demonstrating ferritin blood test results in donors have been published. Retrospective trials with outcomes obtained during 10 and more years are of the greatest interest. Among donors with high rate of donations, 9.4% of males and 25.7% of females had low ferritin levels. An increased donation interval (up to 6 months in males and 8 months in females) results in low risk of iron deficiency [15]. Meanwhile, authors assess iron deficiency depending on gender, age, postmenstrual period, quantity and rate of donations in donors of whole blood only. They, however, fail to assess the values in platelet donors. Thus, it seems relevant to assess the effect of donation type (including mixed donations), donation rate, age, gender and donor experience on the values of iron exchange due to a higher volume of highly specialized medical aid and, as a consequence, whole blood and platelet concentrate banking.

The purpose of the study is to assess the principal values of iron exchange in donors of blood and (or) blood components at risk for developing latent iron deficiency.

METHODS

174 donors of blood and blood components (101 males and 73 females) at the age of 19-62 years (median of 35 years) were investigated. Inclusion criteria: age ≥18 years, weight over 50 kg, readiness to sign an informed consent form (ICF) and refusal from participation in other clinical trials. To examine iron exchange in donors, six groups were formed depending on donor experience, rate and type of donations (blood, platelets, mixed donations for those who donate whole blood, plasma and platelets for four and over years on a constant basis). All patients were divided into groups according to gender and age. Donors were distributed into three groups: under 25 (students), 25 to 45 (regular donors, middle group) and above 45 years (active donors). A group consisting of 130 blood donors was isolated to determine an effect produced by a number of donations on a donor's body. Donors were recruited and examined as specified in regulatory documents. Exclusion criteria: temporary or constant contraindications to blood donation established on the day of assumed donation as per regulatory documents [16]. Hematological, biochemical and statistical methods of research were used in the work. A set of reagents (Coulter LH Series Retic PAK Reagent Kit; US) (Roche Diagnostics GmgH; Germany) was utilized to estimate iron exchange. Hemogram values were assessed using the Medonic M-Series (Boule Medical AB; Sweden) Hematology Analyzer, medical devices registered under the established order (S-Monovette vacutainer tubes 2.6 ml K2EDTA labeled as REF 04.1901.001 (Sarsted AG Co.KG Germany); microtubes 1.5 ml, Sarsted, Eppendorf type, 39*10.8 mm with RR graduation, neutral with Safety cap (Sarsted AG Co.KG; Germany). Serum ferritin was examined to assess iron stores in donors by immunoturbidimetric technique. Concentration of transport iron was analyzed based on serum iron (SI), serum transferrin (ST), total (TIBC) and unsaturated iron-binding capacity (UIBC) of serum and such an estimate as Transferrin Saturation Index (TSI). Cobas Integra 400 plus Biochemistry Analyzer (Roche Diagnostics; Switzerland) was used to perform studies. Soluble transferrin receptors (sTfR) were determined using automated immunochemistry analyzer (Beckman Coulter LH Series; Coult USA company) by immunoenzyme technique. Statistical analysis was done using SPSS 24.0 program (Dell; USA). The obtained results were represented as a median, first and third quartiles. Mann-Whitney test was used to assess significance of parameters between the groups. Intragroup differences were assessed using pair-wise comparison and Wilcoxon test. Differences were considered statistically significant when the probability of error was not exceeding 0.05 ($p < 0,05$).

RESULTS

It was found out that 174 donors distributed into six groups depending on the type, rate of donation, gender and age, blood picture values were almost similar to reference values. During assessment of intense changes in spare and transport iron values, 228 abnormal values from the reference range were analyzed (Table 1).

Comparative analysis of examination results of the principal values of iron metabolism in the investigated donors has shown that the level of ferritin is the most informative value. Levels of ferritin below the reference values were seen in donors of all groups, except for primary male donors (Figure).

Low ferritin levels below the reference values were seen in 39 of investigated males of 101 (38.6%). Depleted iron stores were detected in 32 of investigated females of 73 (43.8%). Level of ferritin, which identifies the absence of body iron stores (less than 12–15 μg/l), was seen in 14 male donors (13.9%) and 19 female donors (26%).

Borderline values of Hb were seen in 19.8% of regular donors of blood and blood components ($n = 174$). It was 119 g/l

Table 1. Factors of latent iron deficiency in different groups of donors of blood (blood components)

Risk factor of latent iron deficiency	HGB, low borderline	SF Normal value	SF ↓ Latent iron deficiency	SI ↓	ST ↑	TIBC ↑	TSI	sTfR ↑	Total deviations
Deviations from reference values	30 (17.2%)	103 59.2%	71 (40.8%)	25	10	41	36	15	228
TYPE of donation:									
Primary	4	26	3	2	–	–	2	–	11
Donations 1–2 times during a year	5	13	11 (45.8%)	4	1	7	7	3	38
Regular, every 3 years	4	10	5 (33.3%)	2	3	9	5	2	30
Regular, every 4 and more years	10	21	30 (58.8%)	9	3	14	12	8	86
Mixed donations	3	13	6 (31.6%)	4	1	4	5	–	23
TCP donors	4	20	16 (44.4%)	4	2	7	5	2	40
Gender of donors:									
Males	10 (26.3%)	62	39 (38.6%)	14	7	22	21	9	122
Females	20 (66.7%)	41	32 (43.8%)	11	3	19	15	6	106
Age of donors:									
Younger than 25 years	8	21	14 (40.0%)	6	1	8	10	2	49
25–45 years	18	61	46 (43.0%)	10	5	23	18	9	129
Over 45 years	4	21	11 (34.4%)	9	4	10	8	4	50

in three females (1.7%) from various groups only. Donors with Hb values at the lower limit of normal (130 g/l in males and 120 g/l in females) with deviations of 3–6 g/l and donors of thrombocytapheresis (TCP) often have a tendency to depletion of iron stores in case of continuous subsequent donations and are, consequently, at risk of latent iron deficiency [13]. Hb values at the lower limit of normal and low SF levels were detected in 30 (42.2%) of 71 donors. Borderline values of Hb and threshold values of ferritin (30 μg/l in male donors and 20 μg/l in females) were risk criteria for iron deficiency (Table 2).

The group at risk of iron deficiency included 58.3% of young female donors who gave their blood 1–2 times per year and 54.4% of female apheresis platelet donors (Table 3). The risk of early latent iron deficiency was detected among male (66.6%) and female (50%) donors who gave blood during four and over years on a regular basis. Mean value of ferritin in male donors was 27.37 μg/l, which is below the reference values (30.0 μg/l).

The values of iron exchange were analyzed in 130 blood donors to detect the effect of the number of donations on iron deficiency. Low SF was noted among three investigated female donors within the control group (primary donors, 28 people) during the first donation. Following the second donation, female donors (n = 11) had an increased level of sTfR (4.28 ± 0.26 g/l), TIBC and UIBC in a significant drop of ferritin (17.38 ± 3.2 μg/l). The reasons can include significant changes in the values of iron exchange during the first year of donation, which are particularly pronounced among female donors. It is known that females have less iron stores in the body (35–40 mg/kg) as compared to males (50 mg/kg body mass) [17]. The third blood donation was followed by a progressive drop of SF concentration among male donors with a subsequent increase of sTfR and TIBC. It is established that iron stores gradually decrease with increased donation intensities. This is particularly notable for the concentration of SF in males. According to the studies, a significant decrease of SF (28.1 ± 4.4 μg/l; n = 28) below reference values (30.0–400.0μg/l) was detected among male donors after ten blood donations. The changes are less evident in female donors. This is probably associated with an increased interval between donations. Low level of SF was found after the second blood donation

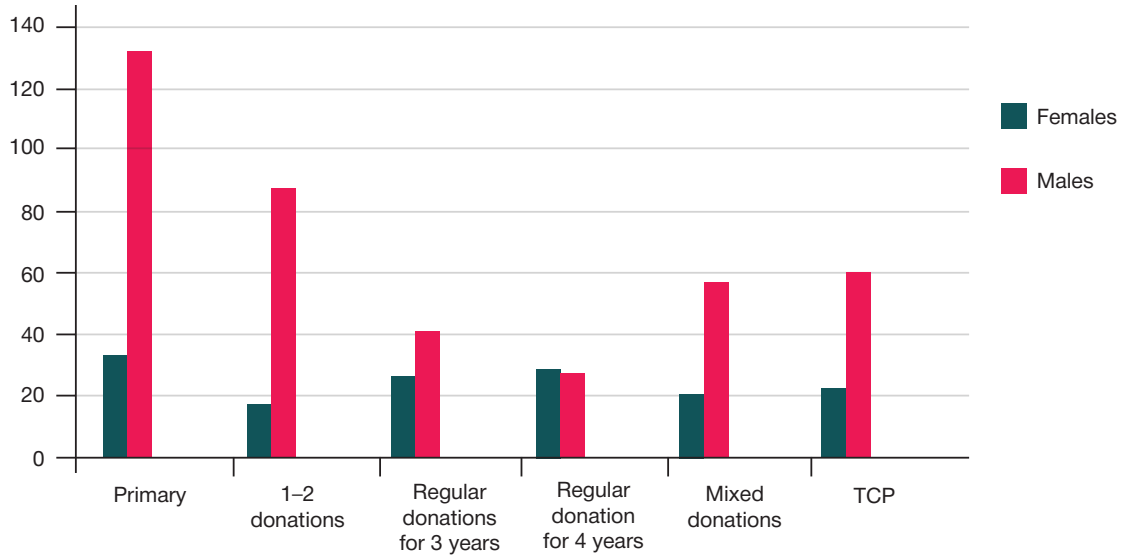

Fig. Changes in the level of serum ferritin (μg/l) among donors from the investigated groups

Table 2. Risk criteria of latent iron deficiency in different groups of blood (blood component) donors, (M ± SD)

Value	Primary (control)	1–2 times a year	Regular 3 years	Regular 4 years and >	Mixed donations	TCP
Females	*n* = 14	*n* = 12	*n* = 6	*n* = 24	*n* = 6	*n* = 11
Ferritin, μg/l	33.3 ± 4.5 (9.3–65.9)	**17.38 ± 3.2* (3.5–37.2) *p* = 0.0042***	26.8 ± 5.4 (14.5–47.8)	28.9 ± 3.5 (9.00–77.4)	**20.3 ± 5.1* (2.4–34.3) *p* = 0.007***	22.8 ± 5.13 (9.6–55.3)
HGB, g/l	131.2 ± 1.9 (120–144)	131.1± 2.8 (121–150)	133.0 ± 4.6 (117–146)	130.9 ± 1.8 (119–153)	132.2 ± 3.9 (121–145)	128.7 ± 2.3 (121–132)
Males	*n* =15	*n* =12	*n* = 9	*n* = 27	*n* =13	*n* = 25
Ferritin, μg/l	132.3 ± 24.5 (33.3–379.0)	88.2 ± 34.0 (8.5–296.0)	41.7 ± 9.9 (13.0–101.8)	**27.37 ± 3.02* (7.2–72.0) *p* = 0.0014***	57.8 ± 8.9 (14.8–122.0)	60.9 ± 8.77 (5.8–177.9)
HGB, g/l	154.3 ± 3.83 (128–168)	148.7 ± 2.8 (132–164)	142.9 ± 4.1 (130–167)	146.5 ± 1.9 (132–170)	150.6 ± 2.3 (134–163)	147.7 ± 1.53 (128–158)

Note: * $p < 0.01$ — statistical significance in the group of primary donors.

with a subsequent significant drop below the reference range. This is the basis for determination of SF during examination of donors after the second and every tenth blood donation. Thus, borderline allowable values of Hb and (or) HCT prior to blood or platelet donation (↓ in 30 people), number of donations (6–10) [13] and duration of donor experience (3–4 years) [13] produced an effect on iron metabolism in donors. Depleted iron stores were seen among young female donors between the second and sixth donations, and among male donors with 10 donations and more.

Thus, periodic control of SF level is required for timely diagnostics of aberration of iron metabolism, including in case of normal content of Hb in blood. The reason for iron deficiency in donors of blood and blood components is the loss of certain amount of iron during every donation and its slow restoration from the incoming food [18]. During donations, donors have to consider an issue about prevention of iron deficiency to replenish the iron depot in the body. Signs of LID will require preventive activities and, if necessary, an increased interval between donations. This will promote preservation of a donor capacity. Preventive activities that can decrease the risk of LID are shown in Table 4.

DISCUSSION

Iron deficiency is a serious threat to donor potential. In accordance with the obtained data, latent iron deficiency in donors is developed due to duration of donor experience and short intervals between donations. To preserve donor potential, donors are examined to detect depleted iron stores. Common ferritin and alternative values of iron exchange (transferrin, soluble transferrin receptors) can be used as markers. All donors with borderline Hb level, female donors of a reproductive age after the second donation and males with ≥ ten donations have to measure the levels of SF. The basic principles of LID treatment include correction of reasons, which form the basis of iron deficiency, and elimination of iron deficiency in blood and tissues [14, 15].

According to our data, the level of SF below the reference range requires correction of this value due to an increased interval between donations and intake of iron preparations. However, the treatment strategy can result in lower stores of donor blood components at blood transfusion centers (blood banks). Thus, an increased interval between donations resulted in lower stores of donor blood by 8% in the first year. In five years, the value was 4.7% [19]. A number of donors with iron deficiency and anemia dropped by 13.6% and 29.3% respectively. The treatment strategy produced a slight effect on blood stores (–3.2% in 5 years). In our opinion, this is a long-term approach. In 10 years, it will allow to return to initial values of donor blood stores, increase the stores, and improve the quality of erythrocyte-containing components.

Thus, it is reasonable to have ongoing monitoring over donors with an increased number of blood donations per year by a number of necessary parameters of iron exchange and borderline Hb value and take a decision regarding the increased duration of an interval between donations or regarding a limited allowable number of donations per year.

When the donor experience is increased in four and over years, the rate of LID within the group of investigated donors is progressing. This prevents iron deficiency and stores donor's health. Iron deficiency is mainly the issue

Table 3. Groups of donors of blood and (or) blood components which are more prone to the risk of iron deficiency

Groups of donors of blood and (or) blood components	Lab value
Regular male donors Donor experience: ≥ 3 years Age group:< 25 and over 45 years	Donations: ≥ 6 HGB > 130 g/l SI ≤ 9.0 μM/l; SF ≤ 29.0 μg/l
Regular male donors Donor experience: ≥ 4 years Age: 25–45 years	HGB > 130 g/l Donations: ≥ 10 SI ≤ 9.0 μM/l; SF ≤ 29.0 μg/l
Female donors who gave their blood 1-2 times a year Age: 18–25 years	Donations: ≥ 2 HGB > 120 g/l SI ≤ 9.0 μM/l; SF ≤ 20.0 μg/l
Female donors mixed donations Age: 18–25 years	Donations: ≥ 6 HGB > 120 g/l SI ≤ 12.0 μM/l; SF ≤ 19.0 μg/l
Female donors thrombocytapheresis Age: over 45 years	Donations: ≥ 10 HGB > 120 g/l SI ≤ 9.0 μM/l; SF ≤ 19.0 μg/l

Table 4. Preventive activities reducing the risk of iron deficiency

Donors at risk of LID	Strategy of reducing the risk of iron deficiency in donors
Donors aged < 25 years Donors with frequent donations (> 3 times per year for males and > 2 per year for females) Donors with Hb values close to the lower limit of normal (within 135 g/l for males and 125 g/l for females) Donors with ferritin values below the reference range are ≤ 20 μg/l for females and ≤ 30 μg/l for males	1. Increased interval between donations (for instance, ≥ 6 months if no iron preparations are taken) 2. Measurement of ferritin as the basis for the motivation of donors to an independent increase of intervals between donations or recommendation of iron preparations

of nutrition. Thus, an adequate and balanced diet at any age constitutes primary prevention of iron deficiency conditions and latent iron deficiency. It is important to diagnose iron deficiency even in the lack of clinical signs, inform donors of consequences and select an optimal drug in every case by using the personalized approach [20, 21]. It is necessary to develop new programs of rational diagnostics and prevention of iron deficiency by using drugs with high effectiveness and good tolerance, which allow to replenish iron stores in LID. Preventive activities for depleted iron stores allow to preserve health of donors and reduce the rate of exemption of donors from donation in repeated blood donations and, thus, to preserve donor potential.

CONCLUSIONS

The conducted studies confirm that the complex assessment of iron exchange is necessary during the first medical examination of donors to allow for access to blood and blood component donation in order to detect latent iron deficiency and preserve health. Timely detection of latent signs of iron deficiency and risk factors of anemia belong to the most important aspect. Donors with multiple blood donations require to assess the processes of iron exchange as the rate of LID increases. As the issue of iron deficiency in donors is pressing, assessment of Hb level and introduction of serum ferritin study into the extensive practice of donorship can be of a great preventive value.

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PATTERNS OF ACUTE CHEMICAL POISONINGS IN A METROPOLIS AGAINST THE BACKGROUND OF THE COVID-19 PANDEMIC IN 2020–2021

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The spread of COVID-19 in Russia has led to restrictive measures. The stress associated therewith had a noticeable psychoemotional effect on the population, which could not but affect the numbers and patterns of acute chemical poisonings (ACP). This study aimed to investigate the patterns of ACP in Moscow in the context of the COVID-19 pandemic. We analyzed data describing cases admitted with ACP to N.V. Sklifosovsky Research Institute for Emergency Medicine in 2019–2021, factoring in the dynamics COVID-19 prevalence as diagnosed with RT-PCR tests. The results of the analysis were processed using nonparametric methods and GraphPad Prism 9 software. Within the considered period, 2020 was the peak year. The number of acute poisonings (AP) with ethanol and its surrogates in 2020 was 109.7% greater than in 2019 (both sexes; the figure for women alone was 286.2%). Male patients suffered AP with drugs and corrosive substances more often than female ($p < 0.0001$). The number of drug abuse cases in 2019–2021 varied slightly, increasing by 2.4 and 6.7% annually. Synthetic narcotic substances were most common: methadone, cathinones, psychostimulants, and mixtures of substances. We discovered parallel trends in dynamics of ethanol intoxication and COVID-19 cases, and no such between drug poisonings and the said morbidity. Thus, the identified specifics of ACP patterns in the capital of Russia associated with the COVID-19 pandemic are a spike in alcohol abuse (especially among women), and lack of noticeable effect of the disease on use of drugs.

Keywords: Poisonings, substance abuse, COVID-19, drugs, methadone, alcohol, ethanol, medicines

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СТРУКТУРА ОСТРЫХ ОТРАВЛЕНИЙ ХИМИЧЕСКОЙ ЭТИОЛОГИИ В МЕГАПОЛИСЕ НА ФОНЕ ПАНДЕМИИ COVID-19 В 2020–2021 ГГ.

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Распространение COVID-19 в России обусловило проведение ограничительных мероприятий. Связанная с ними стрессовая ситуация оказала заметное психоэмоциональное воздействие на население, что не могло не отразиться на эпидемиологии острых отравлений химической этиологии (ООХЭ). Целью исследования было изучить структуру ООХЭ в Москве в условиях пандемии COVID-19. Проанализированы данные обследования лиц, поступивших с ООХЭ в НИИ СП имени Н. В. Склифосовского в 2019–2021 гг., с учетом динамики выявляемости COVID-19 методом ОТ-ПЦР. Для статистической обработки результатов использовали непараметрические методы и программное обеспечение GraphPad Prism 9. В 2020 г. количество госпитализированных с ООХЭ было наибольшим за анализируемый период. По сравнению с 2019 г. число острых отравлений (ОО) этанолом и его суррогатами в 2020 г. у лиц обоего пола возросло на 109,7%, у женщин — на 286,2%. У мужчин чаще ($p < 0,0001$) регистрировали также ОО наркотиками и разъедающими веществами. Число случаев ОО наркотиками в 2019–2021 гг. менялось незначительно, увеличиваясь на 2,4 и 6,7% ежегодно. Преобладали синтетические наркотические вещества: метадон, катиноны, психостимуляторы, а также смеси веществ. Выявлены соответствие тенденций помесячной динамики интоксикаций этанолом с выявляемостью COVID-19 и отсутствие такового при отравлениях наркотиками. Установлены характерные особенности структуры ООХЭ в столице на фоне пандемии COVID-19: рост числа ОО, связанных со злоупотреблением алкоголем (особенно у женщин), при сравнительно стабильном уровне ОО, обусловленных наркопотреблением.

Ключевые слова: отравления, злоупотребление алкоголем или наркотиками, COVID-19, наркотики, метадон, алкоголь, этанол, лекарственные средства

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In the early 2020, a new severe acute respiratory infection, COVID-19 (CoronaVirus Disease 2019), caused by the SARS-CoV-2 coronavirus, entered the Russian Federation and rapidly spread therethrough. The country's capital, being a logistics and transport hub, was one of the first locations to see imported cases and a sharp increase in the incidence of COVID-19 [1, 2]. In March, Moscow imposed restrictions aimed at preventing spread of the new coronavirus infection: citizens were forbidden to leave their places of residence (stay) and told to observe social distancing [3].

The forced self-isolation, characterized by drastically fewer social contacts, and much less active habitual social and physical activities, had a significant stressful effect on the population [3], including vulnerable groups thereof, comprised of, inter alia, drug addicts and people suffering from anxiety and depressive disorders [4-6]. The resulting traumatic conditions could not but affect the patterns of acute chemical poisonings (ACPs). In this connection, investigation of the character and frequency of acute poisonings (APs) in the capital metropolis during the new coronavirus infection spread was deemed to be a relevant task.

The purpose of this study was to investigate the patterns of chemical poisonings in Moscow the context of the COVID-19 pandemic.

METHODS

This is a retrospective cohort study assessing the results of chemical-toxicological analysis of samples taken from patients admitted to the acute poisonings and somatopsychiatric disorders department (APSDD) of N. V. Sklifosovsky Research Institute for Emergency Medicine (Sklifosovsky Institute) in 2020–2021. To create a comparison dataset, we analyzed similar cases (APs, presumably associated with COVID-19) of 2019.

In ACP cases, laboratory diagnosis included 2 stages: preliminary, which employs immunochromatographic assay and thin-layer chromatography, and confirmatory, which uses liquid chromatography with mass-selective detection enabled by SCIEX QTRAP 6500+ (Sciex; USA) to detect phenazepam (benzodiazepines), synthetic cannabimimetics, and derivatives of cathinone, and gas chromatography enabled by Agilent 7890B with mass-selective detector 5977B (Agilent Technologies; USA), Agilent 7820A with mass-selective detector 5975 (Agilent Technologies; USA), to detect other substances.

The study included citizens with various types of AP admitted to the Sklifosovsky Institute via the emergency room and the reception ward. Persons that refused hospitalization were excluded from the study. All AP cases were ranked according to the main nosologic groups according to ICD-10 (Table 1). We analyzed cases of poisoning with individual toxic compounds, medicines, drugs, psychotropic substances, and combinations thereof. APs with illicit stimulants, such as amphetamine (methamphetamine), were considered intoxication with psychotropic agents (ICD-10 class T43, T43.6 - Psychostimulants with abuse potential).

The patients were tested for SARS-CoV-2 RNA by reverse transcription- polymerase chain reaction (RT-PCR), using a set of reagents registered in the Russian Federation. Nasopharyngeal and oropharyngeal swabs served as biological material for molecular studies. Data for the retrospective analysis of COVID-19 incidence were taken from the unified city medical informational and analytical system (ALISA).

Detectability, the ratio of the number of positive SARS-CoV-2 tests to the total amount of tests made within a certain period (as a percentage), was used in collation of the ACP and COVID-19 cases admitted to the Sklifosovsky Institute.

The results were processed using GraphPad Prism 9 (GraphPad Software; USA). The data is given as absolute (n) and relative (%) values. The trends of the frequency of ACP cases with COVID-19 in the background were established with the help of moving average. The relationship between COVID-19 cases registered in the Sklifosovsky Institute and in Moscow in general was determined using the Spearman's rank correlation coefficient. In the context of analysis of attributes, we looked into the frequencies of their occurrence by building contingency tables and applying the Pearson's chi-squared test. The differences were considered statistically significant at $p < 0.05$ (95% probability).

RESULTS

From 2019 to 2021, 9590 patients sought medical assistance at APSDD of the Sklifosovsky Institute (Table 1).

To compare the dynamics of admittance with ACPs (and the respective etiological patterns) to the Sklifosovsky Institute with the specifics of spread of coronavirus infection in Moscow, we analyzed the overall rate of detection of COVID-19 in people admitted in 2020-2021 (Table 2, Figures 1, 2).

Previously, it was established that SARS-CoV-2 morbidity in the capital of Russia has two seasonal spikes [7], which is consistent with data from the concurrent epidemiological studies [2]. A comparative analysis has shown that detection of SARS-CoV-2 RNA in all patients admitted to the Sklifosovsky Institute reflected the COVID-19 epidemic process in the metropolis perfectly: the correlation with the screening of Moscow's population (data collected at the city's clinics and hospitals of various profiles) was very high, Spearman's $r = 0.8402$, $p < 0.0001$ [7]. Thus, data on the COVID-19 cases in the Sklifosovsky Institute can be used in the analysis of ACP patterns in the context of the general epidemiological situation associated with the pandemic (Fig. 1, 2).

Table 1. Patients with ACPs by main etiological groups

Etiological groups of toxicants	ICD-10 code	Studied period (year)					
		2019		2020		2021	
		Abs.	%	Abs.	%	Abs.	%
Medicines	T36-39, T41-50	1642	50.7	1389	39.9	1377	48.1
Drugs	T40	583	18	597	17.1	637	22.2
Alcohol, organic solvents, aromatic and non-aromatic hydrocarbons	T51-T53	434	13.4	910	26.1	242	8.5
Corrosive substances	T54	324	10	349	10	267	9.3
Other	T55-T65	257	7.9	240	6.9	342	11.9
Total	–	3240	100	3485	100	2865	100

Table 2. Dynamics of COVID-19 detection among patients of the Sklifosovsky Institute, years 2020–2021

Month	Time of PCR testing for COVID-19					
	2020			2021		
	SARS-CoV-2 RNA detection results					
	Number of tested patients	Number of positive tests		Number of tested patients	Number of positive tests	
		Abs.	%		Abs.	%
January	–	–	–	4985	652	13.1
February	–	–	–	4262	359	8.4
March	–	–	–	5052	422	8.4
April	1031	354	34.3	4598	393	8.6
May	2406	524	21.8	4107	363	8.8
June	4526	345	7.6	5042	705	14
July	4102	87	2.1	4646	518	11.2
August	3981	139	3.5	3958	253	6.4
September	4490	209	4.7	4359	291	6.7
October	6987	889	12.7	5056	683	13.5
November	5906	910	15.4	4758	536	11.3
December	6537	1009	15.4	4990	325	7.2
Total	39966	4466	11.2	55313	5500	9.9

The age of those admitted with acute intoxication ranged from 16 to 96 years, with male patients and young people prevailing among them throughout the entire period covered by this study (Tables 3, 4).

From the perspective of etiology, acute poisoning with medicines prevailed among the reasons for admittance to Sklifosovsky Institute's APSDD, with most such patients being female (Table 5). In 2019 and 2021, the proportion of such poisonings in women, among all the acute intoxication cases, was largely the same, whereas in 2020 it decreased noticeably. The number of female acute alcohol (and its surrogates) poisoning cases, on the contrary, has increased significantly (by 286.2%) in 2020 compared to 2019, and in 2021 it dropped down again.

Within the entire analyzed period, the etiological patterns of ACPs in women remained largely the same. They sought medical assistance at Sklifosovsky Institute's APSDD because of acute intoxications with prescription medicines, including dormitives and sedatives, antidepressants, neuroleptics, spasmolytics, antiparkinsonians medications, taken, in some cases, with alcohol and/or drugs. The most commonly identified drugs were psychodysleptics, psychostimulants, diacetylmorphine (heroin), and synthetic opioids — methadone, fentanyl, and tramadol.

Overall, men had similar medicines behind their acute poisonings. However, unlike women, they exhibited no spikes in respective numbers: the share of medication-induced acute intoxications has been decreasing steadily in relative and absolute values, with the drop in 2021 against 2019 equaling 22.4%.

Gender-related differences were observed for other types of toxic agents, too. Men were significantly more often ($p < 0.0001$) diagnosed with APs caused by drugs, alcohol and its surrogates, corrosive substances, etc. (Table 5).

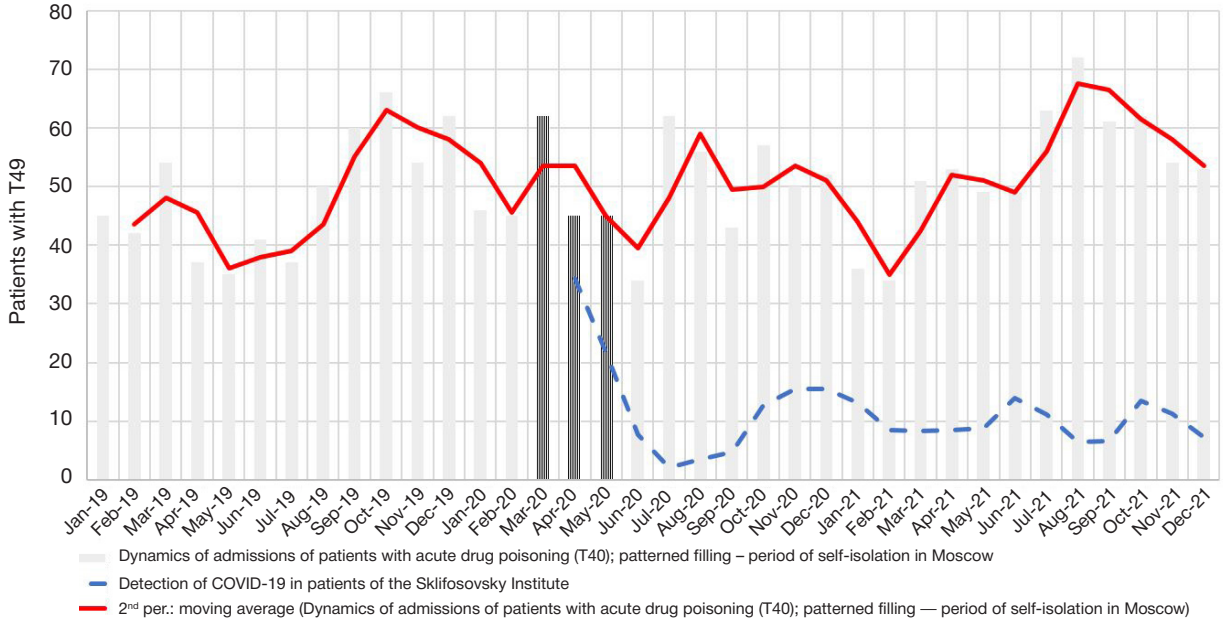

Fig. 1. Dynamics of admissions of patients with acute drug poisoning (T40) in 2019–21, and detection of COVID-19 in 2020–21 among patients of N. V. Sklifosovsky Research Institute for Emergency Medicine

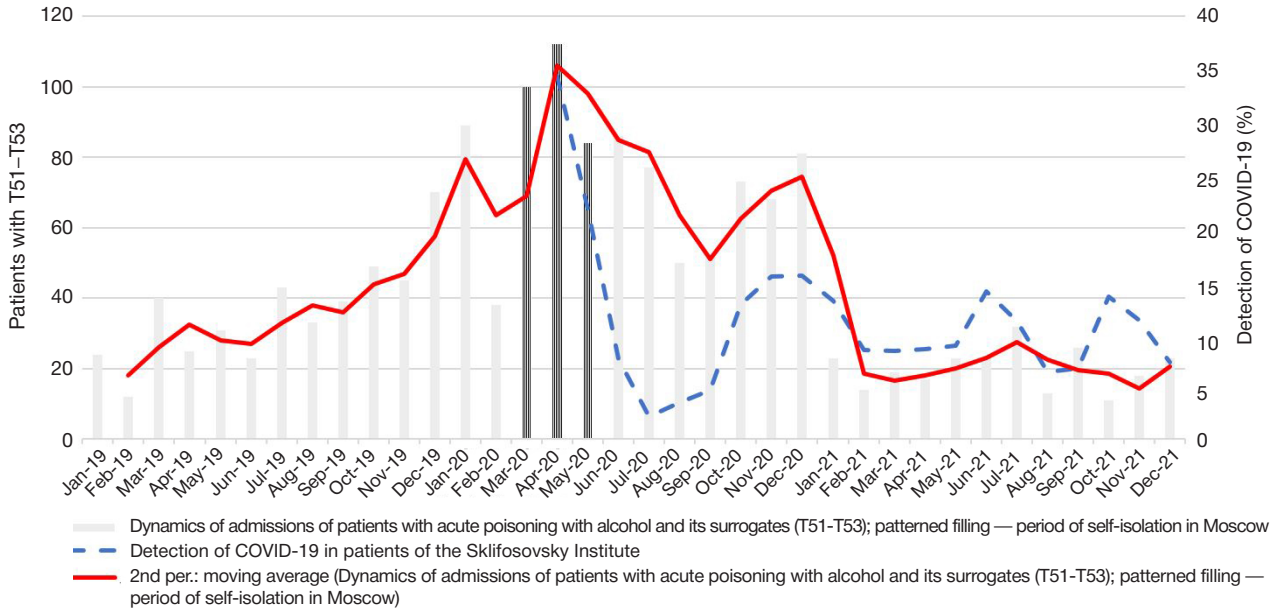

Fig. 2. Dynamics of admissions of patients with acute poisoning with alcohol and its surrogates (T51-T53) in 2019–21, and detection of COVID-19 in 2020–21 among patients of N. V. Sklifosovsky Research Institute for Emergency Medicine

With the COVID-19 pandemic in the background, the situation with drug-related APs has changed significantly from the viewpoint of range of substances abused, while the absolute number of cases remained largely stable. In 2020, the number of drug poisonings that required admission to the hospital has grown by only 2.4% compared to 2019. In 2021, the upwards trend continued, but the rise was still only slight (by 6.7% compared to the previous year) (Table 1).

During the entire study period, synthetic opioid methadone (T40.3) was the most frequently detected drug (Fig. 3). It was the prevailing reason of poisonings in men, with 148 patients hospitalized in 2019, 173 in 2020, 143 in 2021. In this group, acute intoxications with methadone were 1.5–2.4 times more common than with other opiates/opioids. As for women, there were only 17, 33, and 21 cases registered in the considered years, respectively.

The drugs detected in female patients most often were psychodysleptics (T40.9). Men also sought medical assistance because of intoxication with psychodysleptics, and the number of the respective cases doubled through the study period (48 cases in 2019, 76 in 2020, 101 in 2021).

It should be noted that intoxications solely with methadone were rare: 4.2–5.0% (men) and 0.3–0.8% (women) of all drug-induced APs. All other cases involved other drugs, ethanol and/or substances from different pharmacological groups.

Compared to 2019, in 2020 the share of APs with methadone and psychostimulants, psychostimulants/psychodysleptics, and medicines increased from 8.5 to 11.6% and from 6.3 to 13.7%, respectively. The proportion of intoxications with psychodysleptics in combination with opiates/opioids (excluding methadone) and psychostimulants increased from 7.4 to 9.7%, and poisonings with combinations of psychodysleptics and psychostimulants — from 8.3 to 10.2%. The share of APs caused by a combination of methadone and medicines, psychodysleptics and cannabis/psychostimulants, psychodysleptics and medicines, including cases with involvement of ethanol, dropped in 2020, but increased again in 2021. New combinations of toxicants not registered in the previous years were recorded in 2021: opiates/opioids with medicines; synthetic drugs with medicines and/or psychodysleptics and/or cannabis; cocaine with psychostimulants and/or medicines (Fig. 3).

From 2019 through 2021, the overall proportion of AP cases involving a mixture of different substances has grown by 44.2%, but the gender-wise distribution of this rise was very unequal: 0.6% for men, 152.8% for women. As a rule, the mixtures included drugs combined with one or more psychotropic or multidirectional medicines, or with alcohol. Quite often, NSAIDs (sodium metamizole, ibuprofen, naproxen, salicylates, paracetamol) and/or psychotropic medicines (barbiturates, benzodiazepines, tri- and tetracyclic antidepressants) were found combined with drugs. Overall, through the study period, the frequency of registration of intoxications with combinations of drugs and medicines in men increased by 6.6% (Table 5).

The shares of APs with opioids (heroin, morphine, codeine (T40.0-T40.2)) taken alone or in a complex combination of drugs (excluding methadone) and psychopharmacological medications, including T43.6 (derivatives of amphetamine and methamphetamine), varied during the study period from 11.0 to 18.5% (108 cases in 2019, 110 cases in 2020, 70 cases in 2021). In 2019 and 2020, such intoxications were registered in men exclusively, but in 2021, women appeared in the respective group of patients, with these kinds of poisonings making up 13.3% of all cases.

In 2020 and 2021, COVID-19 epidemic process did not influence the monthly dynamics/number of admission of patients with drug-induced APs (Fig. 1). Moreover, when the frequency of detection of SARS-CoV-2 RNA decreased, which

Table 3. Dynamics of acute poisoning, men and women, years 2019–2021

Gender	2019		2020		2021	
	Abs.	%	Abs.	%	Abs.	%
Male	1721	53,1	1988	57	1473	51,4
Female	1519	46,9	1497	43	1392	48,6

Table 4. Age of patients with ACPs admitted to the Sklifosovsky Institute's APSDD

Age group	2019		2020		2021	
	Abs.	%	Abs.	%	Abs.	%
16–29 years old	944	29,1	1014	29,1	926	32,3
30–39 years old	884	27,3	1044	30	772	27
40–49 years old	612	18,9	668	19,2	485	16,9
50–59 years old	347	10,7	333	9,5	265	9,3
60–74 years old	271	8,4	262	7,5	239	8,3
≥ 75 years old	182	5,6	164	4,7	178	6,2
Total	3240	100	3485	100	2865	100

indicated a temporary improvement of the epidemiological situation, the number of such intoxications increased sharply, reaching the maximum in July–October 2021.

During the study period, 2020 was the year when the number of cases of intoxication with alcohol and its surrogates spiked (109.7% more than in 2019 and 2021), and this reason became more common in the overall patterns of ACPs (Table 1).

In 2020, on the level of months, there were 2.5-4.5 times more admissions for this reason than in 2019; the respective indicator spiked in March and April, same time when the number of COVID-19 registrations was maximum (Fig. 2). In 24.8–31.4% of cases (880 persons in 2019, 729 in 2020, 651 in 2021), patients with poisonings of various etiology, with the exception of group T51-T53, were also in a state of alcoholic intoxication.

The number of APs with corrosive substances peaked in 2020 (Table 1). However, in 2021, the respective figures decreased significantly, both in absolute and relative values. In this group, the prevailing patterns were oral intake of organic (acetic) and inorganic (sulfuric, hydrochloric) acids, alkalis (ammonia, sodium hydroxide), oxidants (potassium permanganate, iodine), and corrosive substances part of household chemicals. There were also cases of poisoning with chlorine vapors.

In 2021, compared to the means recorded in 2019 and 2020, the quantity of intoxications with primarily non-medical substances (groups T55-T65, "Other") increased by 33.1%, which translated into growth of their share in the overall ACP patterns (Table 1). The most common reasons for poisonings were carbon monoxide (31.1–39.2%) and toxic substances contained in mushrooms (13.6–29.2%). Cases of the latter kind were registered throughout the year, predominantly during summer and autumn.

In 2020 and 2021, compared to 2019, the number of hospitalizations with toxicological trauma caused by poisonous plants increased 4-fold, from 13 cases in 2019 to 50 and 53 in the following years, respectively. These injuries were mainly seasonal, registered in spring and summer, with photochemical dermatitis (burns) caused by Heraclium sosnowsky being the most common: their proportion varied from 72 to 100% within the studied three years.

DISCUSSION

ACP is a serious public health problem, one of the frequent causes of admission to emergency rooms [8, 9] and mortality in working age [10, 11].

Although far from all persons suffering intoxications of various etiology seek medical assistance, analysis of prevalence and patterns of APs based on the records from multidisciplinary hospitals of metropolises yields valuable information that

Table 5. Etiology of ACPs, men and women admitted to the Sklifosovsky Institute's APSDD

Year	Etiological groups	Male		Female		Statistical analysis results, 95% CI
		Abs.	%	Abs.	%	
2019	Medicines (T36-39, T41-50)	604	18.6	1038	32.1	$p < 0,0001$ ($\chi^2 = 466,7$, df = 4)
	Drugs (T40)	471	14.5	112	3.5	
	Alcohol and its surrogates (T51-T53)	340	10.5	94	2.9	
	Corrosive substances (T54)	166	5.1	158	4.9	
	Other (T55-T65)	140	4.3	117	3.6	
	Total	3240 (100%)				
2020	Medicines (T36-39, T41-50)	553	15.8	836	24.0	$p < 0,0001$ ($\chi^2 = 421,9$, df = 4)
	Drugs (T40)	500	14.3	97	2.8	
	Alcohol and its surrogates (T51-T53)	641	18.4	269	7.7	
	Corrosive substances (T54)	180	5.2	169	4.9	
	Other (T55-T65)	114	3.3	126	3.6	
	Total	3485 (100%)				
2021	Medicines (T36-39, T41-50)	469	16.4	908	31.7	$p < 0,0001$ ($\chi^2 = 407,6$, df = 4)
	Drugs (T40)	502	17.5	135	4.7	
	Alcohol and its surrogates (T51-T53)	177	6.2	65	2.3	
	Corrosive substances (T54)	131	4.5	136	4.7	
	Other (T55-T65)	194	6.8	148	5.2	
	Total	2865 (100%)				

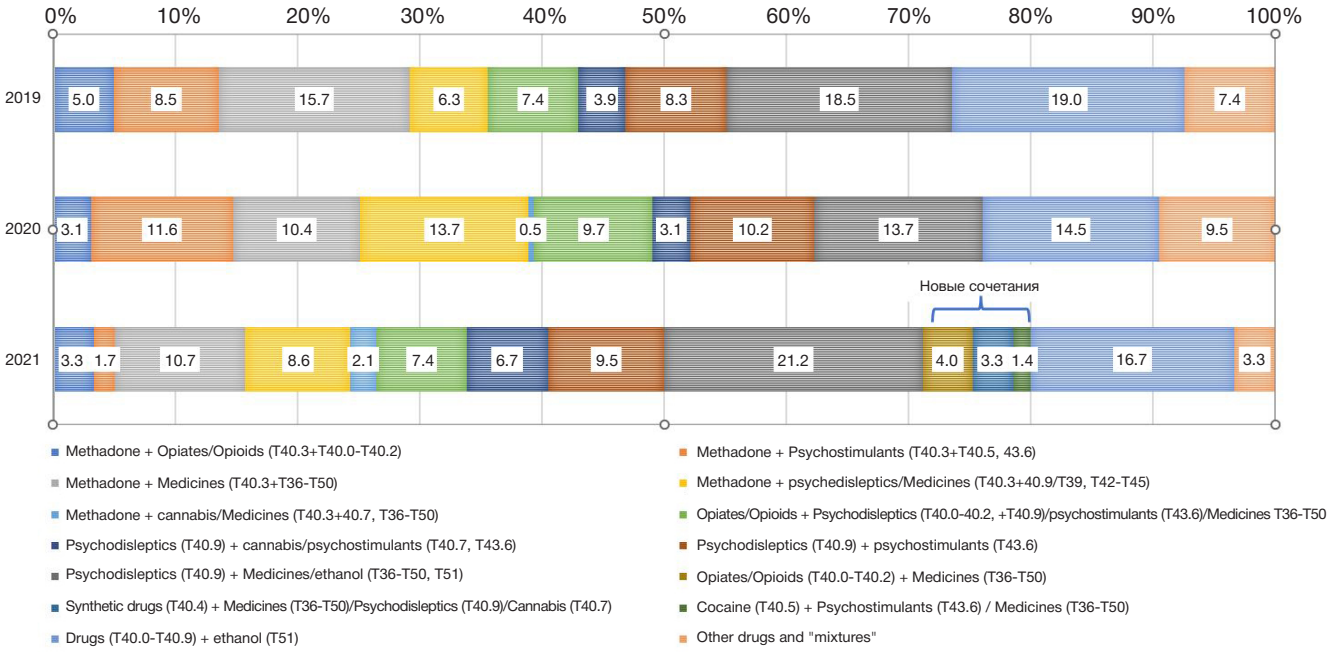

Fig. 3. The proportion of poisonings with drug mixtures

allows deducing trends and regularities peculiar to this branch of medical toxicology [8, 12]. Therefore, it was interesting to investigate the degree and etiological patterns of ACPs against the background of a complicated sanitary and epidemiological situation associated with the COVID-19 pandemic.

Fluctuations of COVID-19 incidence caused, inter alia, by the emergence of the new genetic variants of SARS-CoV-2, and the respective rise and fall of the hospitalizations curve were registered in Moscow and generally in Russia both in 2020 and 2021 [2, 13]. However, from the point of view of population's socio-psychological adaptation to the sanitary and epidemiological situation, the most difficult was 2020, when the imposed restrictive measures were most stringent, and the level of psycho-emotional stress highest [5, 14].

Our study revealed distinct differences in the dynamics of admissions to the Sklifosovsky Institute with toxicological trauma throughout the year preceding the COVID-19 pandemic, then when it was at its highest point, and afterwards, when the epidemiological situation has stabilized. Our data on the number of laboratory-confirmed cases of the new coronavirus infection among those admitted to the hospital allowed objectifying information about the spread of infection in the population during the study period.

Compared to 2019 and 2021, the frequency of referrals to the Sklifosovsky Institute's APSDD with ACPs in 2020 was considerably greater, which is a noteworthy fact. Another interesting aspect in this context is the growth of hospitalizations with APs caused by ethanol and its surrogates. In absolute values, the peak thereof was registered during the first months of sanitary restrictions. This sharp growth of the share of patients with alcohol poisoning probably stems from the high level of psychological stress [5, 15] caused by movement restrictions imposed due to the COVID-19 pandemic, and, apparently, from the widespread opinion that drinking strong alcohol reduces the risk of contracting colds [16].

Intoxication with isopropyl alcohol, a result of abuse of alcohol-containing liquids intended for sanitary treatment of hands and surfaces, also shaped the general patterns of alcohol poisoning. As reported in the published studies, against the background of the COVID-19 pandemic, many countries registered unusually numerous complaints connected with poisoning with disinfectants [17].

On the other hand, there may be another reason behind the increased number of admitted patients with APs caused by ethanol and its surrogates: when COVID-19 was spreading, the process of rendering emergency medical care in Moscow was adjusted to challenges. Thus, some of the inpatient clinics that previously received such cases were completely or partially repurposed to work with COVID-19 patients (under Orders № 44 of January 30, 2006, № 349 of April 5, 2020, № 392 of April 10, 2020, № 584 of June 4, 2020, all issued by the Moscow Department of Health and in force during the study period), which largely diverted the flow of AP cases to the Sklifosovsky Institute.

However, such a redistribution of the said ethanol/surrogates AP cases between Moscow's hospitals should have mainly affected the absolute number of hospital admissions. This is exactly what happened in St. Petersburg, Russia's second largest metropolis, where I. I. Dzhanelidze Research Institute of Emergency Care marked a decrease in the number of referred alcohol poisonings because of the changes in the conditions of hospitalization to medical institutions of the city during the pandemic [18].

At the same time, regardless of the absolute number of admitted patients, the apparent coincidence of the peaks of hospitalizations and COVID-19 detection that occurred in both 2020 and 2021 indicates a spike in alcohol abuse against the background of the pandemic, which is confirmed the moving averages calculated with a smoothing interval of two (Figure 2). This phenomenon may be explained by the psychogenic factor rooted in the population's constant awareness of the morbidity dynamics and the gradual tightening of restrictive measures. This hypothesis is further confirmed by a considerable drop in the absolute number of hospitalizations with this type of poisonings in 2021, as compared to 2020: many restrictions had been canceled in Moscow during the second year of the pandemic, regardless of the still high incidence [2, 19]. In addition, the dependence we have established is consistent with the data of some foreign researchers, who also registered abnormally higher numbers of alcohol poisonings in 2020 [20, 21]. The factor of stress can also be behind the increased proportion of female patients in the 2020's alcohol APs pool: in adverse conditions, women are more likely to develop various

affective disorders, like reactive depression, generalized anxiety and panic disorders [22].

A particularly interesting subject was that of the effect of COVID-19-associated stress and restrictive measures on the patterns of acute drug poisonings in the metropolis.

Both the absolute number of such intoxications and their share in the overall patterns of ACPs remained stable throughout the study, showing only a slight growth by 2021. During the two years of the pandemic, neither the total number of patients admitted with drug poisonings nor the undulating fluctuation thereof through the year have shown any dependency on the COVID-19 incidence rate (Fig. 1), which makes the dynamics of drug-induced APs within the considered period totally different from that of acute intoxications caused by alcohol.

To a certain extent, a probable reason behind the growth of the number of drug poisonings is the involuntary social isolation and the related stress, which turned people to drugs [5], and some of them continued using them afterwards. On the other hand, people who used drugs irregularly before COVID-19 could reduce or even stop taking them during the pandemic, while regular users, on the contrary, could increase doses and/or frequency [23]. In this case, we would have witnessed more intoxication cases requiring hospitalization. Anyhow, it is obvious that sanitary and epidemiological situation has a significantly lower effect on drug abuse than on alcohol overindulgence, since spirits are a more affordable, legal "corrector" of the psycho-emotional status.

Against the background of the pandemic, drug use patterns have changed more qualitatively than quantitatively, with an upwards trend for simultaneous consumption of several narcotic and psychotropic substances. Such mixtures, detected with the help of laboratory tests, indicate either a "falsification," when the initial drug is diluted with other substances, or a switch to an "alternative" preparation with the aim of relieving the withdrawal syndrome [24–26].

Throughout the entire study period, we have also registered a consistently high proportion of "pharmaceutical addiction" cases, i.e., people suffering intoxication with official medicines taken either alone or in combination with alcohol [27].

In 2020, the number of poisonings with natural (morphine, opium) and semi-synthetic (heroin) opiates decreased significantly, by 33.3%, which was probably caused by disruption of the supply chains carrying these drugs along the "Balkan route" from Afghanistan and Pakistan due to the closed borders between the countries [28]. The admitted cases of APs with synthetic narcotic substances and their various combinations with other drugs, medicines and ethanol, on the contrary, have spiked during the pandemic (Fig. 3).

It is obvious that, despite the complete or partial lockdown in different countries, drug users were able to quickly adjust to the difficulties of trafficking. Illicit substances were actively purchased through specialized Internet websites and delivered in a contactless manner [29]. It is likely that the growing frequency of use of synthetic drugs, and, consequently, their rapid spread among consumers, have been supported by their lower cost compared to the traditionally used narcotics.

In the context of the pandemic, the dynamics of referrals to the Sklifosovsky Institute's APSDD with APs of a different genesis has also changed.

It seems quite understandable that, at the height of the pandemic, the number of poisonings with corrosive substances increased, since they could be used for the purpose of additional disinfection. The cases of APs with chlorine vapor have also become more frequent. This type of ACPs, resulting from improper use of disinfectants, was especially common in the first months of the pandemic [30].

There are obvious reasons behind the surge in hospitalizations with toxicological injuries caused by poisonous plants. Because of the switch to remote work patterns and the need for isolation, summertime, many residents of Moscow left for the country, where the possibility of contact with plants is much higher. The prevalence of photochemical dermatitis among phytotoxicoses is the consequence of the continued invasion of Sosnovsky's hogweed, the plant behind such conditions [31].

The stress caused by the spread of COVID-19, with changes of the usual way of life in the background, has been shown to worsen chronic somatic and endogenous mental disorders, the aggravation manifested as insomnia, anxiety, depression [6]. Attempts at arrest thereof often lead to uncontrolled intake of various drugs and dietary supplements. From 2020, we have been registering a growing number of intoxications associated with microdoses of psychedelics contained in fly agaric (Amanita muscaria) or panther amanita (Amanita pantherina) [32], a consequence of the so-called agaric microdosing.

According to the Sklifosovsky Institute, mushroom poisonings are no longer limited to summer and autumn, as they previously were, when the only cause thereof was consumption of poisonous mushrooms by mistake.

CONCLUSIONS

The dynamics of admissions with ACPs to the Sklifosovsky Institute's APSDD during the first two years of COVID-19 differ distinctively from those seen in the year before the pandemic. Apparently, the greater number of alcohol-induced APs is connected with the level of psycho-emotional tension and stress against the background of a complex sanitary and epidemiological situation. The restrictive measures designed to upkeep social isolation do not affect the level of drug use in Moscow fundamentally, but change the respective etiological patterns. The pandemic is associated with an increased number of APs caused by consumption of mood-enhancing substances (psychostimulants, ethanol, agaric microdosing) and use of agents possessing or deemed to possess a disinfecting effect. The data collected at the emergency care hospitals can help identify the actual ACP trends peculiar to a metropolis.

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POSSIBILITY OF USING SUBMENTAL FLAP FOR LOWER LIP RECONSTRUCTION

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Head and neck reconstruction surgery is a challenging area of surgery that requires the surgeon to be familiar with various reconstructive options. Achieving both functionality and aesthetic harmony of facial proportions constitutes one of the most important aspects of the head and neck defect elimination. For that various methods are used involving application of local, regional and free flaps on vascular pedicles. The reconstructive method is selected based on the defect size, location, composition, as well as on the age, comorbidity, surgeon's and patient's preferences. Submental flap is a regional flap that has proven to be a reliable fasciocutaneous flap, the tissues of which are identical to that of the lower face in width, texture, and color. Long vascular pedicle ensures wide flap rotation arc, thereby allowing one to use the flap for elimination of defects of the upper and lower lips, mental region, tongue, floor of the mouth, and preauricular area. Damage to the donor site is minimal, it is cosmetically invisible due to the scar hidden in the mental region. The paper presents the results of surgical treatment of the 38-year-old female patient with the soft tissue defect of the lower third of the face and the lip resulting from trauma. The wound did not heal for more than six months, no improvement was observed. It was decided to eliminate the defect using a rotation submental flap. The patient was followed up for a year after surgery. We managed to achieve complete aesthetic and functional rehabilitation of the patient.

Keywords: submental flap, lip defect, regional flap, maxillofacial defects, reconstructive surgery, microsurgery, plastic surgery

Author contribution: Danishchuk OI, Nazarian DN — surgical procedure, manuscript writing and editing; Karpova EI — surgical procedure; Khachatryan AA — manuscript writing; Razmadze SS — patient management, manuscript writing.

Compliance with ethical standards: the informed consent to case study publication was submitted by the patient.

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ВОЗМОЖНОСТИ ПРИМЕНЕНИЯ ПОДПОДБОРОДОЧНОГО ЛОСКУТА ДЛЯ РЕКОНСТРУКЦИИ НИЖНЕЙ ГУБЫ

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Реконструктивная хирургия головы и шеи — сложная область хирургии, требующая от хирурга владения различными реконструктивными опциями. Одним из важных аспектов устранения дефектов головы и шеи является достижение не только функциональности, но и эстетической гармонии пропорций лица. Для этого применяют различные методы, включающие использование местных, регионарных и свободных лоскутов на сосудистой ножке. Выбор реконструктивного метода зависит от размера, локализации, состава дефекта, возраста, сопутствующей патологии, предпочтений хирурга и пациента. Субментальный лоскут — это регионарный лоскут, который зарекомендовал себя как надежный кожно-фасциальный лоскут, ткани которого идентичны таковым нижней зоны лица по толщине, консистенции и цвету. Длинная сосудистая ножка обеспечивает широкую степень ротации лоскута, что позволяет применить его для устранения дефектов верхней и нижней губы, подбородочной области, языка, дна полости рта и предушной области. Ущерб донорской области минимален и косметически незаметен за счет скрытого в подбородочной области рубца. В статье представлен результат хирургического лечения 38-летней пациентки с дефектом мягких тканей нижней трети лица и губы, который был получен в результате травмы. Рана не заживала больше шести месяцев, положительная динамика отсутствовала. Было принято решение устранить дефект ротационным субментальным лоскутом. Послеоперационный период наблюдения за пациентом составил год. Нам удалось добиться полной эстетической и функциональной реабилитации пациента.

Ключевые слова: субментальный лоскут, дефект губы, регионарный лоскут, дефекты челюстно-лицевой области, реконструктивная хирургия, микрохирургия, пластическая хирургия

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Соблюдение этических стандартов: от пациента было получено добровольное информированное согласие на публикацию клинического случая.

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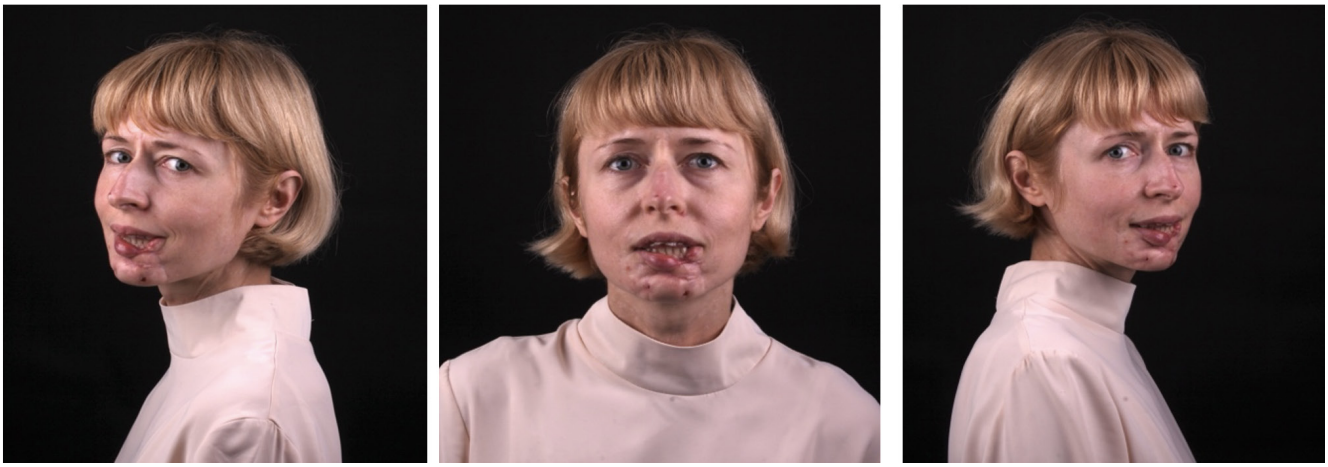

Fig. 1. Anthropophotometry on the day of treatment

Maxillofacial defects have a significant effect on the patients' health and quality of life. Defects of this region result primarily from injuries of different etiology, tissue resection following surgical procedures on resection of masses of different origin, blast injuries, congenital anomalies, and iatrogenic injuries.

High aesthetic value of facial zone, structural features of maxillofacial region represented by the compactly located vital structures, and functional value of this zone determine the difficulty of conducting surgical procedures involving selection of individual plan in each particular case.

Today, selection of surgical treatment for patients with facial defects implies an integrated multidisciplinary approach involving maxillofacial and plastic surgeons, thereby ensuring optimal morphofunctional and aesthetic rehabilitation of patients.

Here we provide a clinical case of complex multistage surgical treatment of the female patient with soft tissue defect in the lower third of the face (jaw and lower lip) involving the use of submental flap and subsequent local tissue correction.

Submental flap proposed by D. Martin in 1993 was selected due to its popularity among oncologists and maxillofacial surgeons commonly operating head and neck for elimination of defects of the neck, esophagus, tongue, floor of the mouth, upper and lower lips [1–3].

The flap is supplied by the submental artery, after which it was named. The submental artery being a branch of the facial artery is a reliable and consistent blood supply source. The average artery diameter is 1.7 mm. On its way the artery produces 1–4 perforator branches to the skin area of the flap, thereby enabling harvesting the flap with a skin paddle sized 18 cm (length) and 7 cm (width). Venous drainage is provided by the eponymous vein that runs into the factial vein. The average vein diameter is 2.2 mm. The vascular pedicle can be 8 cm long, which enables flap rotation up to the zygomatic arch, thereby covering most possible zones in the middle and lower face [4–5].

The advantages of the flap include reliable blood supply, invisible scar hidden in the neck area, large skin paddle and long vascular pedicle, enabling a wide arc of flap rotation [6].

Meta-analysis involving comparison of using submental flaps and free tissue transfer for elimination of oral defects showed that the use of rotation submental flap was associated with less operative time, shorter hospitalization, fewer perioperative complications [7].

There are multiple case studies, in which the rotation submental flap was used to eliminate various maxillofacial defects. In particular, such flap was used to eliminate the upper lip defect with a very good aesthetic outcome [8]. The flap was applied to eliminate the lower lip defect preserving the oral cavity airtightness [9]. A case study was provided, in which two submental flaps were used for total reconstruction of the lower lip defect resulting from the malignant neoplasm resection [10].

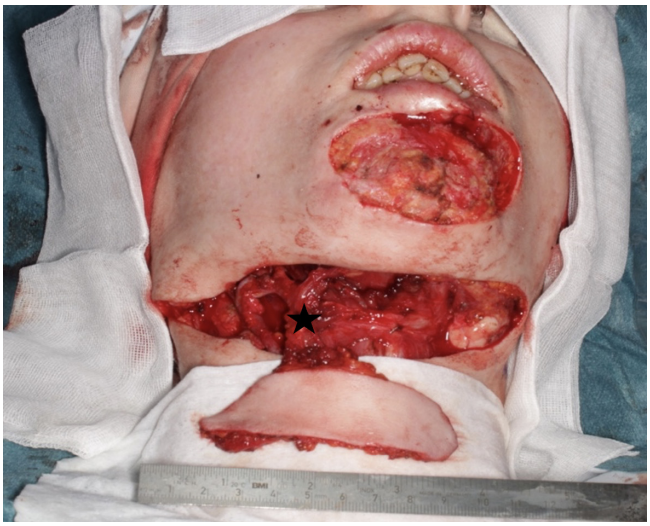

Fig. 2. View of the defect and the harvested submental flap with vascular pedicle (marked with *asterisk*)

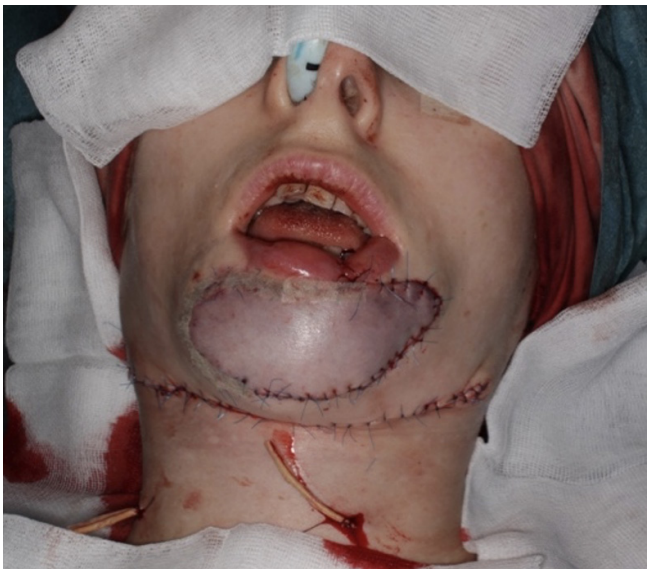

Fig. 3. View of the wound after flap fixation in the defect area and the donor bed suturing

Thus, submental flap is an ideal flap for elimination of facial defects due to texture that is similar to that of facial skin and color match. This can be an excellent alternative to free flaps when used in the head and neck reconstructive surgery [11, 12].

Clinical case

Female patient S., 38 years old, contacted the Department of Maxillofacial Surgery at the National Medical Research Center for Otorlaryngology of FMBA of Russia due to lower lip defect resulting from trauma, non-healing wounds in the chin region (Fig. 1). Histological examination of wound tissues performed in the Center confirmed tissue necrosis and chronic inflammation.

The first stage of surgical treatment involved dissection of necrotic tissue in the mental region and lower lip. To close the resulting defect sized 7 × 3 cm, a submental fasciocutaneous flap sized 8.5 × 2 cm was harvested on the right submental artery and vein (Fig. 2) with subsequent flap rotation through the skin tunnel and fixation in the mental region. The Minidop 8 portable Doppler (Bioss; Russia) was used to identify perforators supplying skin (Fig. 3). The donor region was closed by placing a layer-by-layer suture to form a linear scar that was hardly visible in the submental region.

Venous stasis in the flap formed was observed during the first day. Hirudotherapy was performed for five days in order to improve circulation and reduce venous stasis (Fig. 4). Beneficial effect was reported, the patient was discharged on day 7 in satisfactory condition (Fig. 5).

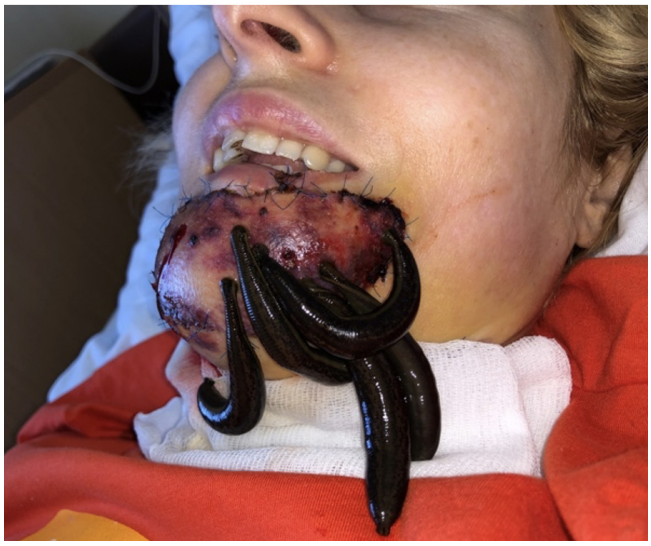

Fig. 4. View of the flap on day three; hirudotherapy is applied

Seven months after the defect closure a residual deformity in the form of cicatricial lower lip shortening and vermillion defect on the left was observed. The second stage of reconstruction involved restoration of the lower lip length/height on the left and elimination of vermillion defect using local tissues. To eliminate the lower lip mucosal defect, we cut a rotation flap via a “rabble” incision along the transitory fold, which was moved into the

Fig. 5. Anthropophotometry: view of the wound on day 7

Fig. 6. Anthropophotometry four months after surgery

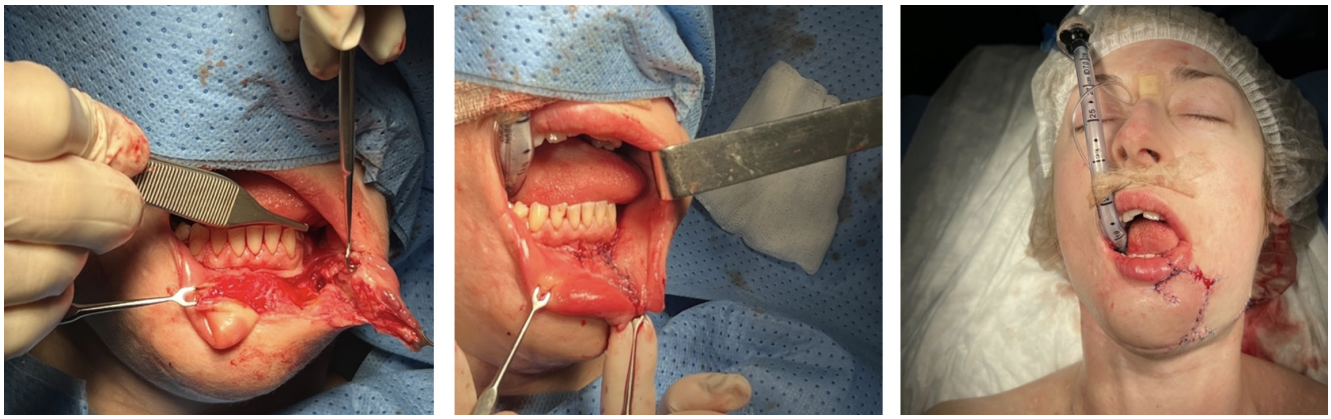

Fig. 7. Second-stage surgery: reconstruction of the lower lip and mental region tissues seven months after the main stage

resulting defect after dissection of mucosal scars. After scar tissue dissection we cut multiple transposable triangular flaps (Z-plasty) from the skin of the lip and chin on the left, which enabled increasing the lower lip length on the left. The vermillion defect was eliminated using the method by Mirault involving cutting a triangular (tongue-shaped) flap from the vermillion border of the lateral lip fragment and a bed for the flap in the medial lower lip fragment. To restore the lower lip function, the remaining orbicularis oris muscle fragments were identified that were sutured by plication (superimposition of fragments). After that sutures were placed layer-by-layer. Stitches were removed on day 10. Wound healing by primary intention took place; no signs of inflammation were observed (Fig. 6, 7).

The patient was followed up for a year after surgery, good aesthetic and functional results were yielded with minimal donor region deformity. The patient could close her lips completely,

Fig. 8. Anthropophotometry 11 months after surgery

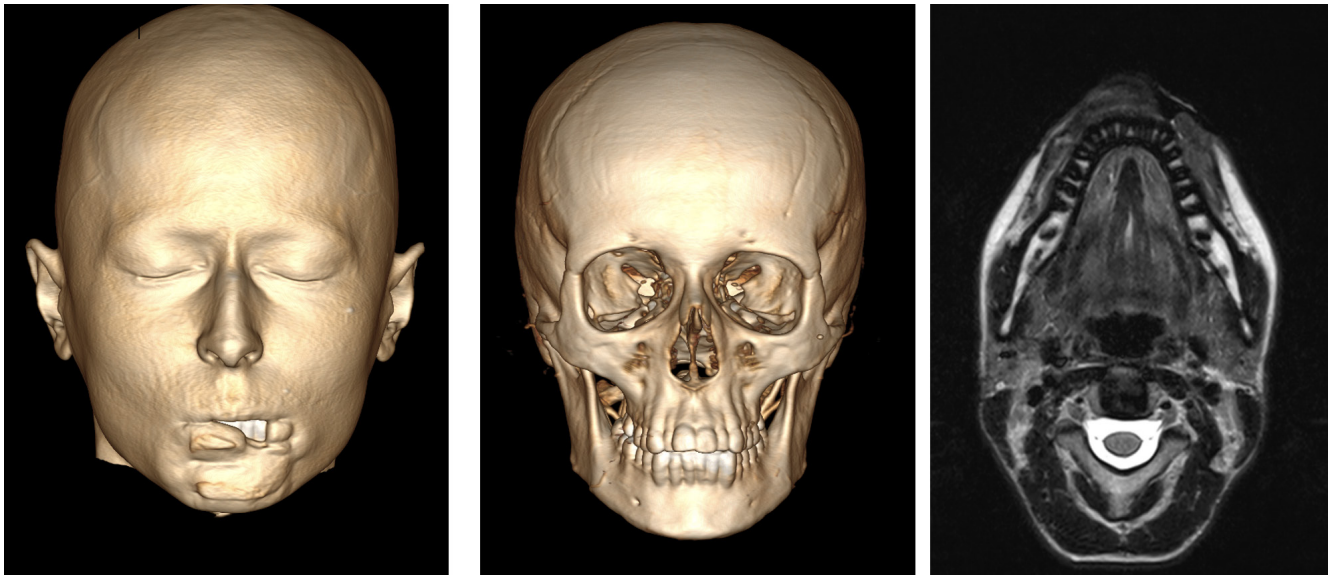

Fig. 9. Contrast-enchanced MSCT and maxillofacial MRI before surgery: no foreign objects are visible in the defect area

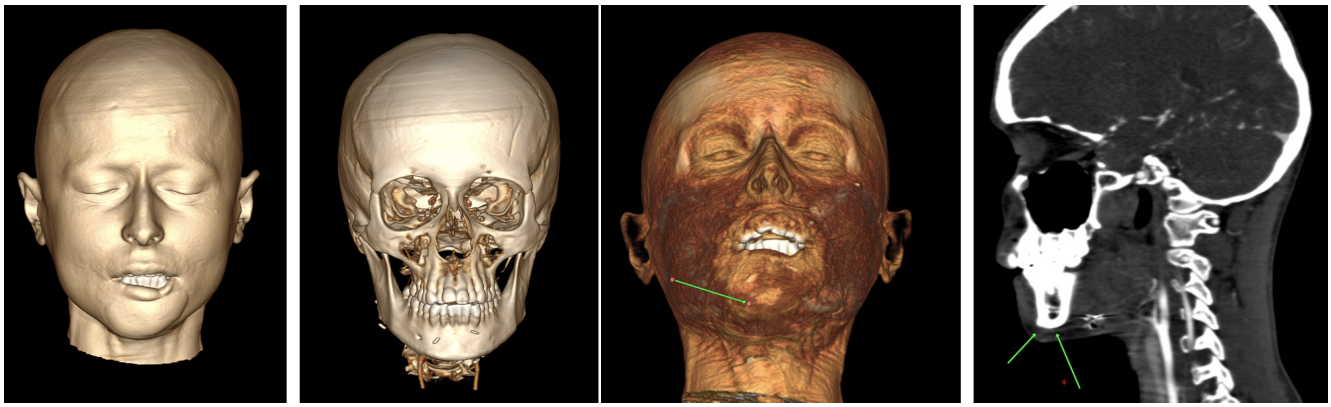

Fig. 10. Contrast-enchanced MSCT after surgery: submental artery is marked with *arrow*

she had no difficulty consuming fluids and foods of any texture (Fig. 8–10).

Clinical case discussion

Various methods for reconstruction of surgical defects of the lower third of the face have been reported. Reconstructive options vary between primary closure and the use of free flaps, depending on the defect size and type [9].

However, for optimal outcome to be achieved, the donor and recipient sites should have similar characterstics in terms of skin quality, thickness, color and texture match. Thus, selection of local regional flap near the facial soft tissue defect is a perfect option [8, 13].

Closure of mental and buccal defects using free flaps and microsurgical technique does not allow one to obtain identical skin color and texture in Caucasian patients when using flaps harvested from the thoracortical, radial, femoral or shoulder areas.

To eliminate residual deformity after the defect closure, supplementary surgical reconstruction with local tissues is required for the patient's appearance improvement.

Advances in microsurgery led to a better understanding of the fasciocutaneous perforator flaps anatomical features, thereby allowing reconstructive surgeons to gain new capabilities of eliminating complex maxillofacial defects [14].

CONCLUSION

Regional flaps are a good alternative to free flaps with vascular pedicles due to less operative time, lower requirements for the patient's somatic status, surgeon's skills, and operating room equipment [7]. This allows one to use flaps of this type in field surgery for immediate elimination of blast and gunshot defects in the lower third of the face.

Long vascular pedicle ensures wide flap rotation arc and the possibility of using the flap for elimination of almost any soft tissue defect of the lower third of the face, while skin characteristics identical to those in the buccal and mental areas make it possible to achieve good aesthetic outcome.

The clinical case reported represents an example of complex approach to surgical treatment of patients with maxillofacial defects involving the use of rotation submental flaps.

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