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MOLECULAR GENETIC STUDIES IN THE CONTEXT OF BIOMEDICAL RISKS FOR COSMONAUTS' HEALTH

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Today, genetic studies yield quite a large amount of information about a person, which, in many cases, allows predicting the risks of certain diseases. This gives grounds to believe that such testing can also be applied in the field of manned spaceflights in order to identify candidates best adapted to specific risks. The article examines publications on genetic polymorphisms and their effects on the carrier phenotype, namely, on such manifestations that are of interest in the context of risks arising during long-term space flights. Specific genes are listed and examples of allelic variants are given. Publications describing new molecular methods of monitoring human health are also considered, biomarkers that can be used for research in the interests of regular examination of active astronauts are identified.

Keywords: genetic predisposition, molecular markers, long-term spaceflight risks, cosmonaut selection

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МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКОЕ ТЕСТИРОВАНИЕ В КОНТЕКСТЕ МЕДИКО-БИОЛОГИЧЕСКИХ РИСКОВ ЗДОРОВЬЮ КОСМОНАВТОВ

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Генетические исследования сегодня позволяют получить достаточно большое количество информации о человеке, на основе которой иногда возможно прогнозировать риски возникновения определенных заболеваний. Это дает основания полагать, что подобное тестирование можно применять и в области пилотируемых космических полетов с целью выявления кандидатов, наиболее приспособленных к специфическим рискам. В статье рассмотрены публикации, посвященные генетическим полиморфизмам и их влиянию на фенотип носителя, а именно на проявления, представляющие интерес в контексте рисков, возникающих во время длительных космических полетов. Перечислены конкретные гены и приведены примеры аллельных вариантов. Уделено также внимание публикациям, описывающим новые молекулярные методы наблюдения за здоровьем человека, определены биомаркеры, которые могут быть использованы для исследований в интересах регулярного обследования действующих космонавтов.

Ключевые слова: генетическая предрасположенность, молекулярные маркёры, риски длительных космических полетов, отбор космонавтов

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With the recent advancements in laboratory diagnostic methods, it is now possible to perform genome-wide DNA sequencing with subsequent analysis of the sequences fairly quickly. Theoretically, knowledge of how each of the alleles in the genotype affects phenotype, individually and in combination with other alleles, allows predicting many important parameters, adaptability to given conditions, predisposition to various diseases, as well as body's response to given influences. It is interesting to investigate applicability of the genetic analysis as the source of data used in the process of selection of candidates best fit for the conditions of spaceflight and least exposed to the risks arising from the associated factors, since such people may have significantly extended professional longevity. However, today, there is only a limited number of alleles with known role in the formation of the phenotype, therefore, it is virtually impossible to obtain complete data that would exhaustively describe characteristics of the body. Still, the amount of available information allows selecting alleles that presumably secure advantages in the context of resistance to the classified and other factors of spaceflight. In addition, there are genes for which the effect of allelic variants on the phenotype has not yet been uncovered. Given that this information may become important in the future, this matter can be addressed additionally. We have also reviewed papers describing molecular studies that we recommend conducting before, during, and after the flight, since they provide the most complete information about both health of the cosmonaut and the specific processes occurring in his body.

Studying allelic variants of genes, we decided to divide them into groups depending on how the considered polymorphisms can mitigate or aggravate risks peculiar to long-term spaceflights. For this purpose, we looked into both Russian and foreign sources, with references to the latter mainly collected while analyzing all the risk evidence documents published to the NASA's Human Research Program (HRP NASA) website. As a result, some of the articles are more

than 10 years old, yet, the authors considered it necessary to include them in the review. We have also relied on additional literature and eventually identified genes whose variations can affect susceptibility of future cosmonauts to factors associated with spaceflight factors, and, consequently, their professional longevity.

Genetic polymorphisms in the context of risks associated with long-term spaceflights

In the context of assessment and mitigation of the risks of development of adverse cognitive or behavioral conditions and mental disorders during spaceflight, there have been identified polymorphisms in the circadian CLOCK and NPAS2 genes, which were shown to trigger sleep disorders [1, 2], one of the factors promoting depression. A significant polymorphism was found in 5-HTTLPR, serotonin-transporter-linked promoter region. Individuals with S-allele have been shown to run a higher risk of depression stemming from routine difficulties and obstacles [3]. Allelic variations in the genes of some ionotropic channels, such as AMPA3 (Gria3 glutamate receptor, ionotropic) or P2RX7 (ATP-dependent selective calcium channel), may increase the risk of appearance of suicidal thoughts against the background of antidepressants; they also aggravate depression accordingly [4, 5]. It was also found that, with a certain haplotype, the methylenetetrahydrofolate reductase gene involved in folic acid metabolism can positively correlate with depressive states [6].

In terms of the risk of productivity and health deterioration as a result of lack of sleep, circadian disorders and overwork, the most common were polymorphisms of circadian genes, such as CLOCK, NPAS2 and PERIOD3, in which certain variations may be associated with sleep disorders and, as in the case of PERIOD3, promote differential neurobehavioral vulnerability to acute total sleep deprivation [7–10]. It was found that catechol-O-methyltransferase (COMT) enzyme, which modulates dopaminergic catabolism in the prefrontal cortex, grows three- to four-fold less active if the amino acid sequence contains Val158Met replacement, which translates into greater availability of dopamine at receptors and a higher concentration of cortical dopamine. This COMT polymorphism predicts less efficient functioning of the prefrontal cortex and poor performance of working memory in healthy subjects with a highly active Val allele [11]. Additionally, in people with a Met/Met genotype the markers of homeostatic sleep pressure decrease more rapidly. With chronic partial sleep deprivation in the background, all genotypes demonstrated a comparable pace of cognitive performance deterioration and physiological drowsiness increase [9, 10]. Polymorphisms in the adenosine dezminase (ADA) gene, adenosine receptor (ADORA2A) gene, and human leukocyte antigen (DQB1) gene are associated with various disorders. The latter was found to condition narcolepsy, a sleep disorder characterized by excessive daytime sleepiness, fragmented sleep and shorter REM sleep delay. Individuals with DQB1*0602 polymorphism of the DQB1 gene suffer a sharper drop of homeostatic pressure during sleep; they are generally more drowsy and prone to fatigue. However, chronic partial sleep deprivation caused comparable decline of cognitive abilities and growth of physiological drowsiness in both carriers and non-carriers of this allele. As it turned out, the adenosine deaminase gene plays a part in alteration of duration of slow-wave sleep, contributing to the interindividual variability of the initial sleep level, and the adenosine receptor gene polymorphism is associated with objective and subjective differences in the effects of caffeine on sleep after acute total sleep deprivation [9, 10].

Regarding the assessment and mitigation of the risks associated with use of ineffective or toxic drugs during a long-term spaceflight, the crucially important factor is the metabolizer status, that is, the rate at which a given individual can metabolize a particular drug. Depending on this rate, such individual may need an abnormal dose of the drug, which can be both smaller and larger. Disregarding peculiarities of metabolism can up the risk of overdoses, or, on the contrary, prevent intake of the drug in the amount needed for it to produce the expected therapeutic effect. Metabolizer status depends on an array of allelic variants of genes that encode enzymes and carrier proteins involved in metabolism and drug clearance.

Enzymes of the cytochrome P450 superfamily play a key role in the metabolism of drugs; they are found in many tissues, but are most common in the liver. The main enzymes from this superfamily are the CYP2D6, CYP2C19, and CYP3A4 proteins. They are involved in processing of most medicines used today; for their genes, there have been identified dozens of alleles that can alter enzymatic kinetics, i.e., the rate of a chemical reaction resulting in either a breakdown or a modification of the drug molecules. Thus, the range of rates at which enzyme isoforms work can be very wide, from almost complete loss of activity to the so-called "ultrafast" variants [12–15].

First-aid kits in the US Orbital Segment of the International Space Station (ISS) contains two types of antidepressants, so the haplotypes conditioning the effects of these drugs have also been considered. The researchers found that some of the allelic variants in 5-HTTLPR and 5HTR6 are associated with a better response to antidepressants, while other, on the contrary, degrade drug tolerance [16, 17].

In the context of assessment and mitigation of the risks related to cardiovascular adaptations, previous studies have uncovered significant polymorphisms associated with various cardiovascular pathologies. Thus, C1561T polymorphism of the GCPII gene is an independent risk factor for coronary heart disease, which makes development of this condition 2.71 more likely, while C1420TT polymorphism of the cSHMT gene almost halves the respective risk [18]. Same group of researchers investigated the MTRR gene (encodes methionine synthase reductase), and found one of the allelic variants in the homozygous state to boost oxidative stress, which also increases the risk of coronary heart disease. The findings also included a genome-wide significant interaction of polymorphisms in the loci of the HCN4 and SLC28A1 genes that is associated with an increased risk of atrial fibrillation [19]. The risk of cardiovascular pathologies is also influenced by the level of low-density lipoprotein cholesterol, as well as the total cholesterol level. A group of researchers established that isoforms of ApoE, a protein involved in the metabolism of fat in mammalian organisms, are linked to fluctuations of the blood cholesterol levels, with the specific values thereof above or below the population average depending on the presence of certain alleles [20].

Regarding assessment and mitigation of the risks of spaceflight-associated intracranial pressure growth and neuro-ocular syndrome, there are noteworthy studies that consider higher blood concentrations of single-carbon metabolites (cysteine, etc.) detected in the astronauts that can develop the said syndrome. Based on the data, researchers assumed that variations in the genes of single-carbon metabolism may increase the susceptibility of astronauts to ophthalmological changes. Eventually, they have found that polymorphisms of the MTRR and SHMT1420 genes significantly condition the effect a prolonged mission to the ISS has on the visual analyzer [21]. Another study has shown that carriers of the MTHFR677TT

polymorphism are more likely to suffer from idiopathic internal hypertension [22].

Searching for genetic variants associated with the risk of radiation carcinogenesis, a team of researchers identified a mutation that occurs in 0.4% of the European population in a heterozygous variant. It is a mutation in the gene of a protein that mutated against the background of ataxia-telangiectasia (ATM, serine/threonine protein kinase, recruited and activated by double-strand breaks); this mutation significantly increases the incidence of breast cancer in women carrying heterozygous variant thereof. Compared to the general population, female carriers of the ATM mutation were also found to be at a somewhat higher risk of cancer in general [23]. Overall, the idea of searching for haplotypes that signal lower susceptibility to malignant tumors looks difficult to implement at the moment, since the nature of the respective diseases is complex and multifactorial. However, in the future, cosmonauts will be sent on long-term missions, those to Mars in the first place, which involve a significantly higher risk of malignant tumors than now. In this regard, the data on preferred haplotypes can be extremely useful, so it is worth continuing investigations of this subject matter.

In addition to those described above, there are also unclassified risks that should still be accounted for. For example, researchers have identified polymorphisms of the Hsp70 (heat shock protein) gene that can both protect the carrier from hearing loss caused by prolonged noise exposure and, on the contrary, increase his sensitivity to this environmental factor [24, 25]. Presumably, Hsp70 is released, inter alia, in response to loud sounds with the purpose of shielding hair cells in the inner ear from damage and subsequent death, but the exact mechanism of protection is still unknown. In addition to noise, space flights imply exposure to radiation; presumably, certain alleles of the apolipoprotein gene [26, 27], and the HLA-DRB1*11 allele of the major histocompatibility complex gene, can protect therefrom to some extent [28].

There are allelic variants of genes the effect of which on the risks associated with spaceflight are yet to be investigated, including various replacements in the catalase (CAT) gene sequence. The polymorphisms identified so far produced opposite effects in different populations, but it is certain that they condition hearing loss significantly [29]. It is also necessary to study genes in which polymorphisms can affect predisposition to sarcopenia, including ACE, ACTN3, MSTN, CNTF, VDR, IGF1 [30]. Finally, there is a link between certain haplotypes of various genes and the rate of progression of osteoporosis that should be looked into. Considering the number of genes involved, osteoporosis is an extremely complex disease [31], and the genetic variants or their combinations that would significantly decelerate the associated bone loss are yet to be identified. Currently, there is an expanding list of candidate genes that can be investigated in this connection [32].

Biomarkers enabling monitoring of a cosmonaut's physical condition indicators

Genotype-based screening of cosmonauts is not the only genetics-related issue in the considered field: there is also a demand for ways to monitor health of the cosmonauts with the aim at extending their professional longevity. The arguments below present biomarkers that, monitored, provide a more complete picture of the physical condition of cosmonauts.

Telomere studies

Presumably, the dynamics of telomere length is an informative biomarker showing the state of health of individuals, including cosmonauts, since it reflects the degree of influence of the factors they are exposed to in space. Individual genetic characteristics, nutritional, psychological, and physical stresses, unique environmental conditions (microgravity, cosmic radiation, altered atmosphere of the space station) — all these factors have an effect on a cosmonaut that can be registered by changes in the length of his telomeres.

Studies show that telomere length, which can be influenced by various lifestyle factors, may signal increased rate of aging and onset of age-related diseases, since it is negatively correlated with age. The expression of biomarkers of telomeric dysfunction and DNA damage, such as stathmin (regulates the dynamics of microtubules; disruptions of its operation may translate into uncontrolled assembly of mitotic spindles) and EF1-a (mediates accommodation of aminoacyl-TRNA into the ribosome), increases with age [33]. Long-term (over 5 years) follow-ups have shown that shorter telomeres mean significantly lower survival rate, which is conditioned by higher incidence of cardiovascular and infectious diseases [34]. DNA instability associated with telomere dysfunction (severe shortening) is an early oncogenesis event. Cancer patients were found to have significantly shorter telomeres compared to the control group [35]. In general, there are many factors that influence the length of the telomeres, gender, lifestyle specifics, diet, psychological load, chronic stress, and illnesses. Telomeres also reflect the effects of the environment on the body, with air pollution, ultraviolet and ionizing radiation having an effect on their length [36]; moreover, they are considered to be distinctive signs of radiosensitivity [37]. Telomeres are difficult to sequence with short reads because they are basically tandem repeats, but recent advancements allowed applying long-read sequencing to them, with results thereof showing telomere length and localization of non-canonical repeats [38].

A study that involved 11 astronauts has shown that, both before and after the spaceflight, their telomeres are shorter, and telomerase less active than in the control group (on Earth), but in the course of the space mission, the length of the telomeres increased significantly. The same study has also revealed a correlation between chronic oxidative stress (peculiar to spaceflight) and dynamics of telomere length, as well as a strong connection linking concentrations of inflammatory cytokines (interleukins, IL4, IL10, IL5, IL1a, IL2), chemokines (CCL5, CCL4, CXCL5), and VEGF-1 and telomere lengths before, during, and after the flight [39]. Throughout the year-long mission to the ISS, astronauts had high blood plasma concentration of VEGF-1, which may be associated with the increased expression of HIF-1a that participates in regulation and activation of hTERT, a human telomerase catalytic subunit [40], thus offering an explanation for longer telomeres during spaceflight.

Exosome studies

Exosomes are extracellular vesicles secreted by cells into the external intercellular space. They contain proteins, RNA, peptides and cell-free DNA. The amount of cell-free DNA (cfDNA) is a dynamic and highly responsive indicator that allows assessing the degree of DNA damage, tumor growth, regulatory changes in RNA, and immune response to infections [41].

cfDNA contain traces of nucleosomes, the nuclear architecture, gene structure, and expression of which yield information about their source tissue. In particular, positioning of nucleosomes may point to traces of transcription factors binding, promoter activity, and splicing, ultimately reporting about the processes of gene regulation in the tissue/cell of origin [42].

One study involved two monozygotic male twins, one of whom spent 340 days on the ISS while another stayed on Earth. The analysis of their cfDNA did not reveal a significant difference in the concentration and distribution of DNA length between brothers, and between them and the control group [41]. However, in the course of the study, the blood level of extracellular mitochondrial DNA was found to have been growing in the astronaut throughout the entire mission. The analysis of exosomes circulating in blood plasma also revealed a high content of ubiquitin-independent proteasome proteins in the spacefaring twin. In addition, the exosomes in his samples contained CD14, a proinflammatory monocyte marker, and basigin and integrin β1, which correlate with development of cancerous tumors and inflammation; the control samples in this study did not have these monocyte and proteins [43, 44]. Moreover, BAIAP2 (brain-specific angiogenesis inhibitor 1-associated protein 2) and BAIAP2L1 (brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1) were identified inside exosomes isolated from plasma samples of the astronaut twin, unlike control samples, which had more proteins associated with regulation of apoptosis and ATP biosynthesis. Three years after the flight, the researchers have registered a correlation between the content of 20S proteasomes and the concentration of exosomes in the astronaut's blood plasma. This protein is an important component of the oxidation-driven degradation mechanism; under oxidative stress, its amount may increase [45, 46]. In addition, a higher content of 20S proteasomes in the exosomal vesicles correlates with pathological processes, such as carcinogenesis, vascular damage, viral infections, and autoimmune diseases. The analysis of plasma exosomes isolated from the astronaut's samples upon his return to Earth revealed a drastically greater amount of circulating particles with untypical types of proteins in them. These changes are unique in comparison with the indicators describing the respective parameters of his twin and the control group of healthy individuals [41]. Since most of the exosomes circulating in plasma originate from immune cells, it is likely that this is a reflection of immune dysfunction associated with spaceflight and subsequent return to normal gravity. The researchers assume that presence of exosomes with brain-specific proteins in the peripheral blood may be the result of alterations in the state of the blood-brain barrier (tight contacts therein) caused by the spaceflight, a phenomenon earlier established for the intestinal epithelial cells [47].

Clonal hematopoiesis studies

Clonal hematopoiesis is faster growth of cells with certain mutations, which ups the risk of hematology and cardiovascular diseases. A study that involved twin astronauts found their blood to contain hematopoietic clones carrying mutations in the TET2 genes (catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine) and DNMT3A (an enzyme that catalyzes the transfer of methyl groups to CpG methylation sites in DNA) [48]. Both proteins are involved in epigenetic regulation; mutations in their genes often disrupt the amino acid sequence, and they accompany hematological cancers [49]. Such mutations usually occur in old age, while in the astronauts examined they were detected two decades earlier than expected. The factors causing early mutations are not known for certain, but they probably stem from the known working conditions on the ISS and associated with spaceflight. Throughout the mission, one of the twin astronauts exhibited signs of vascular remodeling of the carotid artery, which is a fact deserving a special note. He had a mutation in TET2, which also creates a significant risk of cardiovascular diseases. Thus, monitoring of clonal hematopoiesis, along with other parameters, can be included in the comprehensive assessment of the health status of cosmonauts.

Investigation of the effect of spaceflight on the critically important physiological systems of cosmonauts

Of course, for a more complete molecular examination of cosmonauts, it is necessary to consider as many informative markers as possible, thus assessing the state of all bodily systems. A good illustration of the value of such approach is the case of search for the molecules that reflect the state of the cardiovascular system. This search returned detection of higher concentration of S100A9 (neutrophil myeloid protein, important for the regulation of proinflammatory reactions and immune response) in cosmonauts, and this protein is a new predictor of myocardial infarction in patients with acute coronary syndrome. An increased plasma level of the S100A8/9 heterodimer indicates a higher risk of cardiovascular diseases, and it has also been shown that expression of S100A8/9 grows in human atherosclerotic arteries. It is assumed that the S100A9 protein signals damage to the vascular monolayer endothelial cells and induction of proinflammatory reactions in those cells, which are also confirmed when proteins associated with vascular damage and protecting the endothelium are found in blood plasma [50, 51].

Investigations of the effect of spaceflight on the immune system reveal a high degree of variability of the respective indicators, which points to individual predispositions to this or that immunity alteration triggered in space. However, it was reliably established that spaceflight causes the ratio of IFNu/IL10 to decrease; this ratio affects Th1 and Th2 cells, therefore, its disruption can lead to suppression of the immune response. Also, after the mission, cosmonauts had elevated blood concentration of HSP70, a protein massively expressed upon exposure to various stress factors and capable of protecting monocyte-granulocyte cells [52, 53].

Investigation of bone remodeling in cosmonauts revealed that the subjects most sensitive to microgravity have more TRAP than normal in their blood and less OPG therein. These markers allow conclusions about the degree of bone resorption: TRAP reflects the activity of osteoclasts, and OPG is the inhibitor of osteoclastogenesis [54].

Molecular markers associated with damage to the central nervous system (CNS) are also very interesting. A study [55] has shown the effect of radiation exposure on secretion of neurotrophins, composition of cerebrospinal fluid, and metabolism of microRNAs that play a major regulatory role in the nervous system. MicroRNAs can also be found in exosomes secreted by astrocytes, which is important for the CNS's in the intercellular interactions [55].

CONCLUSION

Summarizing the above, we believe it is necessary to note that the above list of molecular genetic markers is not complete, since investigations of the subject matter continue. However, this list considers the most important and well-studied sections of the genome that correlate with human predispositions to certain diseases associated with spaceflight, as well as other biological markers that, monitored, can underpin a more detailed assessment of the cosmonauts' health. Of course, future will bring new data, and the list will have to be expanded. Perhaps, someday, it will be possible to identify haplotypes best fit for space missions, and consequently consider improvement of

the candidate selection process. The given recommendations are primarily aimed at increasing professional longevity of cosmonauts, and there will be more of them. It should be noted that studying these issues is important as part of the Russian space exploration strategy and concept, which involve creation of technological capacities needed for interplanetary flights to Mars and asteroids. Development of the system/means of assessment and mitigation of medical risks faced by crews is integral to this effort. The value of such research increases dramatically in the context of manned deep space expeditions that, compared to low-orbit missions, imply longer exposure to the factors of spaceflight, which have a more intense effect. Thus, the importance of studying the subject matter considered in this work will grow exponentially in the future, therefore, the basis for the respective research activities should be laid in the present time.

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GLOBAL AND NATIONAL BONE MARROW REGISTRIES: EXPERIENCE OF USING, MAIN ISSUES, AND PERSPECTIVES

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The paper provides the summary of foreign literature data on the organizational and methodological aspects of functioning of the bone marrow and hematopoietic stem cell donor registries, the issues of HLA typing, the technical algorithms for compatibility degree ranking. The changes in the citizens' motivations in response to the bone marrow donating program popularization are described, along with the features of arranging recruitment, approaches to determining the requirements for the registry population considering the multinationality and heterogeneity of ethnic composition, and the statistical approximation algorithms. Furthermore, attention is paid to the so-called specific aspects of the functioning of bone marrow and hematopoietic stem cell registries and biobanks. The latter is important in terms of ensuring national security, adaptation of the population to the effects of the disasters, emergencies, and terrorist attacks associated with the development of bone marrow syndrome in a large number of victims.

Keywords: registry, bone marrow, HLA, donors, typing, donor recruitment, transplants, acute radiation syndrome, NMDP, WMDA

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ГЛОБАЛЬНЫЕ И НАЦИОНАЛЬНЫЕ РЕГИСТРЫ ДОНОРОВ КОСТНОГО МОЗГА: ОПЫТ ИСПОЛЬЗОВАНИЯ, ОСНОВНЫЕ ТРУДНОСТИ И ПЕРСПЕКТИВЫ

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В статье обобщен ряд представленных в зарубежной литературе сведений, касающихся общих организационно-методических аспектов функционирования регистров доноров костного мозга и гемопоэтических стволовых клеток, проблематики HLA-типирования, технических алгоритмов ранжирования степени совместимости. Описаны изменения мотивационной сферы граждан в ответ на программы популяризации донорства костного мозга, особенности организации рекрутинга, подходы к определению требований к численности регистра с учетом многонациональности и неоднородности этнического состава и алгоритмы их статистической аппроксимации. Кроме того, уделено внимание так называемым специальным организационно-методическим аспектам функционирования регистров и биобанков костного мозга и гемопоэтических стволовых клеток. Последнее важно с точки зрения обеспечения национальной безопасности, адаптации населения к последствиям катастроф, чрезвычайных происшествий и террористических акций, сопровождающихся развитием у большого числа пострадавших костномозговой формы лучевой болезни.

Ключевые слова: регистр, костный мозг, HLA, доноры, типирование, рекрутинг доноров, трансплантации, лучевая болезнь, NMDP, WMDA

Вклад авторов: О. В. Эйхлер — разработка концепции, написание статьи; А. А. Миролюбов — анализ литературы, написание разделов по специальным регламентам функционирования регистров в условиях возникновения ядерной угрозы, методам поиска начальных источников финансирования национального регистра США; Д. Н. Нечаев — анализ и обобщение количественных показателей работы регистров, оценка мнений зарубежных авторов по проблемам биобанкирования костного мозга и стволовых гемопоэтических клеток; А. Г. Титова — сбор и оценка количественной информации о работе зарубежных регистров; Г. А. Трусов — оценка полноты предоставляемых сведений, помощь в разработке методологии написания статьи; В. В. Гарбуз — анализ принципов построения и работы специальных регламентов функционирования зарубежных регистров доноров костного мозга; А. Н. Ломов — анализ методов и технологий информатизации зарубежных регистров доноров костного мозга; В. С. Юдин — редактирование статьи; А. А. Кескинов — разработка материалов по биоинформатическим методам, применяемым для генотипирования донорского состава зарубежных регистров, редактирование статьи; С. А. Краевой — разработка материалов по организационно-юридическим аспектам функционирования зарубежных регистров доноров костного мозга и гемопоэтических стволовых клеток.

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To date, extensive experience of using the data acquisition and processing systems to facilitate the process of finding the HLA-matched biomaterial for transplantation have been accumulated in the world's healthcare practice. This technological category particularly includes registries of bone marrow (BM) and hematopoietic stem cell (HSC) donors designed to radically increase transplants access facility in terms of a broad range of diseases associated with hematopoietic system abnormalities.

In the context of the steadily increasing diversity of the registered HLA-associated alleles of potential donors, the increase in the likelihood of finding a compatible donor has been achieved, Such kind of circumstances can significantly improve the clinical outcomes of transplantation.

Though the registries of bone marrow donors emerged in different countries in a random-timing manner, we can say with reasonable confidence that currently a 40-year experience of their practical use has been accumulated globally. The wealth of knowledge, achievements, and various methodological approaches accumulated over such a long period could be of use for national medicine, considering the fact that the project to create the Federal Registry of BM and HSC Donors was launched in the RF in 2022. The aim of the paper is to summarize the data related to the organizational and methodological aspects of the register functioning, the issues of HLA typing, and a relationship with the sphere of national security. Certain technical information about the software and hardware arsenal used by the world's registries is important in terms of determining the current competitive position of our country in the field of the information technology support of bone marrow transplantation and innovative advancement reference points delineating.

Organizational and methodological component of foreign registry operation

The registry of bone marrow donors represents a multilevel complex hierarchical system of interaction between the computational algorithms, databases, and information flows from the users' workstations. The use of such systems ensures the coordinated work of diversified medical institutions, a considerable share of which provides highly specialized medical care and high-tech diagnosis. A significant proportion of the themes related to the work of such registries are focused specifically on the biomedical problems of hematology, transfusiology, transplantology, immunology, biotechnological features of determining the polynucleotide sequences, etc. However, the availability of sufficient quantities of high-quality donor material with the target properties and the possibility of its immediate use for transplantation always represent the key problem, no matter how completely all the specialized medical issues are resolved. Solving this kind of problem is related to the need for drafting and subsequent structuring of the organizational and methodological tasks implying accumulation of sufficient quantities of donors with various HLA phenotypes and ensuring the most effective preparation of biomaterial for timely transplantation.

Issues of forming and replenishing the donor pool. Role of motivation

Indeed, the issue of motivation and donors pool replenishment represents one of the most important and fundamental problems of all registries. The reason is that donating bone marrow is associated with several factors, which, at first glance, seem to be a formidable obstacle for involvement of a large human population in voluntary medical activity of this type. First, donating is unpaid. Here it should be noted that, despite the fact that the global issue of medical transplantation commercialization should not be overlooked, the most important national registries forming the basis of the world's medical cooperation in this field work entirely on a gratis basis. Second, donating is associated with feeling uncomfortable and pain, even under anesthesia of any type. Third, there are no close family, friendly, or even social contacts between the donor and the potential recipient. According to the data provided by American authors, the National Marrow Donor Program (NMDP) founders' initial attempts to secure the reliable sources of funding for the wide propaganda of donating bone marrow met with serious resistance that was largely based on the above reasoning. However, in 1991, after using large federal funds for NMDP, the original skepticism dissipated quickly: in just 2 years the donor pool of the registry expanded from 250,000 to 1 million, reaching more than 6 million citizens by the year 2006. The fact of successful consolidation of public opinion around the valuable "altruistic" resource resulted in the need to increase the output, since, according to rough estimates, less than 5% of potential donors attracted to the registry would be de facto activated [1]. In this regard, it was proposed to actively engage registrants in recruitment of donors (by analogy with network marketing, MLM), participation in other medical and public health initiatives, and even in the direct financial support of those. The criticism of such initiatives was overcame by the fact that the registry members initially engaged within the framework of the request for a single donation later agreed to repeat the donation and participate in the other promising form of replenishing the pool of donor material for transplantation, the HSC apheresis associated with the completely different medical manipulations (injections of pharmacological agents followed by the prolonged peripheral venous catheterization for blood collection and reinfusion) [1].

Despite quite expansion of the NMDP registry and the lack of consensus on the method to determine it`s needed number of donors, the importance of various actions and initiatives to involve new participants on annual basis is emphasized by the American specialists, The reasons for that are the problem of "natural decline" in the number of NMDP donors occurring after reaching the age of 61 years and the negative correlation between the donor's length of stay in the registry and the likelihood of his/her successful activation. Based on the accumulated experience, the 4-year threshold of staying in the registry was determined as critical in terms of the sharp increase in the probability of the donor's refusal of activation [2], while it follows from the relatively early NMDP reports that about 30% of the registered donors who match the recipients based on HLA parameters turn out to be not available at the time of activation [3]. Moreover, in 2006, the share of Caucasian recipients, who had absolutely no HLA-matched donors, in the NMDP registry was 25% [1].

The major obstacles to increase the number of registry participants include high demand for funding: adding every 100,000 donors to the registry is associated with additional expenses of up to 10 million dollars [4]. To better illustrate the major changes associated with the multi-fold increase in the number of donors in the registry, it is appropriate to mention that at the dawn of its creation NMDP had only 200,000 donors and one full-time employee, while in 2008, when the number of donors reached 7 million, there were more than 600 employees, and the office area increased to 160,000 m^2 [5].

With regard to the high resource intensity of work on the creation of such registries it is reported that in is necessary to

partially redistribute the material burden, from state to private institutions engaged in charity activities. It is also proposed to work out the measures aimed at reducing the costs of filling the database of the registry donor pool. The potentially useful measures reported include the switch to collection of buccal epithelium biosamples (the method was introduced into practice of NMDP in 2006 [6]), reducing the costs of HLA typing, and improving performance of the recruitment centers [1].

The latter of the above mentioned approaches is being considered by foreign authors from both fundamental point of view involving the analysis of motivational and psychological features of influence on the future donor anurely organizational and methodological point of view confined to describing the features of selection and subsequent professional training of the recruiting staff. Within the framework of the first approach it is reasonable to note the papers focused on studying motivations for entering the registry. It has been found that the priorities and behavioral modes of potential donors represent a complex phenomenon. Furthermore, among factors contributing to making positive decision, altruistic personal traits and social responsibility are noted, while the opposite characteristics include cautious attitude towards healthcare system and adherence to religious values [7–9].

Conducting informational and educational events, as well as introduction of the popularization programs, is considered to be an effective method to deal with the psychosocial features that prevent donating [10–12].

In this context, the studies focused on determining the optimal methods for social communication are of great importance. Thus, the results of the experimental study aimed to compare the effectiveness of rational reasoning and emotion-oriented involvement in the social advertising about transplantation have shown that the emphasis placed on the sentimental component can to the greater extent increase both the likelihood of registration in NMDP and the likelihood of the appropriate information dissemination across family members and close friends of the new potential donor [13].

The practice of accelerated recruitment of the donor pool of the DKMS international registry Chilean branch involves simultaneous use of the whole range of online and offline communication channels, including the official website, information channels of social media, television, radio, press, and news aggregators. Furthermore, the emphasis was placed on the series of one-off actions aimed at helping the specific patient in need of unrelated transplantation. During such actions, which usually took one or two days and involved the patient's parents and friends, people, who were ready to donate bone marrow, were registered at local schools, sports clubs or community centers. As for 2022, a total of 695 such actions had been organized (among them 303 in 2022). The most successful offline event represented a three-day action organized in the cities of Santiago and Temuco, during which more than 6300 potential donors were recruited for the benefit of the 9-year-old female patient with leukemia. Since it was officially founded in February 2018, the registry managed to recruit about 170,000 people ready to donate [14].

The range of recruiting methods used by the Indian Genebandhu registry of bone marrow donors was significantly less diverse and comprehensive: motivational speaking in front of the audience, direct individual-oriented persuasion, hanging banners and posters were used. Furthermore, the registry managed to recruit 7682 potential donors in 2012–2018 [15].

The experience of arranging recruitment of one of the Russian local registries of bone marrow donors, Rosplasma, suggests reliance on the existing network of plasma centers and holding mass actions in educational institutions [16].

The studies of the most common causes of drop-out from the NMDP registry and non-confirmation of the previously declared consent when requested for transplantation revealed predominance of such factors, as changes in health status, discovery of the fact of inadequate clinical assessment at initial recruitment, incorrect registration of contact information, incomplete information about the upcoming procedures and possible complications, etc. [17]. Some authors believe that the category of adversely affecting factors also includes entering the registry together with the individual donating to certain patient (usually a relative) and making the decision for ethnical reasons [2]. The typical reasons of inability to activate potential donors from the Canadian registry include failure of attempts to get in touch using previously collected contact information, inability to donate due to personal reasons, such as interference with work or study, loss of motivation; in 1.8% of cases the registrants refused to specify their "no go" reasons [18].

As for the features of selection and subsequent professional training of the recruiting staff, it is reasonable to divide such specialists into three categories in ascending order of their competence levels: group leaders, professional and volunteer recruiters, in accordance with the World Marrow Donor Association (WMDA) guidelines. Among the skills and characterological profile elements essential for all categories without exception, the ability to build effective communication aimed at boosting recruitment, the ability to maintain and improve contacts with various categories of specialists and registry volunteers, personal empathy and high motivation are noted. It is also noteworthy that, according to the above WMDA document, such a rare option, as effective functioning in multidisciplinary environment, is a keystone trait of the professional recruiter to the registry.

Among the routine functional responsibilities of the specialists under consideration, assessment of the donor availability for activation is particularly emphasized, while among the most critical knowledge, the arguments in favor of the importance of the donor's life saving mission, donor validity criteria and rules for working with confidential information are highlighted [19].

Search algorithms and the likelihood of finding a matched donor

Another important aspect of the organizational and methodological component of the register activity is represented by the timely delivery of the donor material for transplantation, since the time allocated for the search, transportation, diagnostic, legal, financial, and other activities preceding the final phase of hematopoietic failure treatment is usually extremely limited. The NMDP practice suggests partial unification of the search algorithms and strong dependence of the latter on the opinions of the registry physicians and coordination staff. Statistical studies have shown that the approach, in which the first search phase is guided by the hardware algorithm (using the electronic computational system to form the most promising donor-recipient pairs), but the final decision is made by the patient's physicians, is the most popular. The second most important one is represented by meetings of commissions and round tables (meeting of experts presumably using different variants of decision making procedures), while reliance primarily on the hardware algorithm ranks only third. Traxis, NMDP search strategy advice/HLA consultation, HapLogic donor, and CBU match prediction are the most commonly used information resources used for search (in descending order of popularity). Among the main

tools aimed at increasing efficacy of the search made for the benefit of certain patient under time pressure, the following are reported (in descending order of popularity): simultaneous activation and management of several donors, priority setting by the transplantation center coordinator when examining the donors, driving the donor to transplantation in parallel with the process of confirmation typing and limiting the search pool to the donors, the last contact with whom was made recently.

When it is impossible to find the 8/8 HLA-matched donors (match by four most important HLA-associated nucleotide sequences of both chromosomes 6), the strategies related to the search for haploidentical donors, selection of cord blood or even activation of a partially mismatched 7/8 HLA donor are used (in descending order of popularity).

The use of the whole combination of the above measures results in the fact that nowadays inability to find an appropriate donor is not the most serious obstacle on the way to timely transplantation. The far more significant factors include inability of the third-party registry to meet the schedule of biomaterial collection, problems related to acquisition of the typing results, and insurance problems [20].

Despite the perfection and diversity of algorithms for finding matched donors and the steady upward trend in the number of world registries, the issue of their completeness is especially pressing in the context of multinational and ethnically diverse states, where rare variants of HLA phenotypes constitute a significant part of the common pool and turn out to be associated with the closed populations.

A vivid reverse illustration is such country with low HLA diversity, as Japan, where the likelihood of match by antigens A, B, C, and DR of approximately 95% was achieved after the Japan Marrow Donor Program (JMDP) had formed a three hundred thousand pool of potential donors [21].

In Saudi Arabia, the likelihood of finding a 10/10 matched (match by five most important HLA-associated nucleotide sequences of both chromosomes 6) HSC donor is about 50%, given there is a million donors in the registry [22].

A slightly different situation is observed in Israel, where the degree of ethnic and subethnic diversity has a great impact on the likelihood of finding donors with a high degree of compatibility. As for 2017, bioinformatics modeling showed that this parameter was 40–55% depending on the fact of belonging to particular ethnic or subethnic subgroup, and its growth of about 1% per year was predicted based on the registry filling rate [23].

However, the more recent findings suggest that unique alleles have been reported in the sample of 223,960 potential donors added to the Israeli registry in 2018–2021. This fact may indicate that the degree of HLA diversity in the Israeli cohort is still poorly understood and probably should be adjusted upward, which can affect approximation of the likelihood of finding the matched donors [24].

India represents one of the most vivid illustrations of registries filing issues caused by the ethnic diversity of the population. This country is home to more than 300 ethnic groups speaking 438 languages, and the pools of five main registries of bone marrow donors are as follows: DKMS Registry (21,695 donors), Be The Cure Registry-Jeevan Foundation (6449 donors), Datri Blood Stem Cells Registry (367,561 donors), GeneBandhu (7991 donors), and Marrow Donor Registry India (MDRI) (35,768 donors). In this case, the likelihood of finding an appropriate variant for transplantation, even without considering the requirements for high HLA typing resolution, is below 20%, since more than a third of HLA types are unique (the so-called singletons) [22]. Furthermore, it should be emphasized that the probability values provided for India are empirical, while these provided for KSA (Kingdom of Saudi Arabia) are estimates (prognostic values). The key parameter that opens up the possibility of this kind of approximation and, as a consequence, the possibility of estimating the target values of register fullness, is the measure of genetic HLA diversity of the population, which, given practical impossibility of typing 100% of all individuals in the population, also requires statistical prediction. The probabilistic approximation of such type becomes available due to extrapolation to the whole population of HLA typing data of the registry donor pool. The lack of unified typing methods is the main difficulty preventing the mentioned procedure in foreign countries, which results in eclectic picture of HLA data with different resolution accumulated over the decades, along with high abundance of unique alleles (high percentage of singletons). The most obvious and simplest solution to the problem of consistency of information about HLA genotypes, reduction of all data to the lowest resolution of all represented in the system, leads to the significant decrease in the allele diversity recording performance, that is why it has been proposed to use the statistical algorithms capable of operating in the context of samples that are mismatched based on the specified criterion [25]. As for overcoming the second obstacle, it is necessary to note the statistical developments in adaptation of the expectation–maximization algorithms to the distributions characterized by the so-called heavy tails. Application of the algorithm to the data of donors from the US national registry has in particular shown that 44.65% of the haplotypes of Caucasian Americans are singletons, i.e. are unique. Furthermore, the share of representation of the haplotype variant types in the register relative to their total number among Caucasian citizens of the United States is only 23.45%. However, the 6.59 million pool of donors is enough to ensure 99.4% population coverage due to the fact that 90% of Caucasian Americans have one of the common haplotypes (4.5% of cases) [26].

Thus, the targets of the number of donors are calculated using mathematical modeling, the results of which depend heavily on the characteristics of input data, specifically HLA typing data. These characteristics may change depending on the method of reduction to one or another standard and, therefore, affect the mathematical model performance. Hence, we can conclude that the issues of filling the registry and calculating the target values of this parameter are rather closely related to the issues of the applied HLA typing methods' standardization.

HLA typing in activities of the world's registries

Currently, there are no data on using the standardized approaches to HLA typing in the international peer-reviewed literature. The multivariate nature of the HLA genotype determination procedures persists in several areas at once, which merits special consideration.

Completeness of information about the nucleotide sequences of genes encoding the major histocompatibility complex (MHC) proteins obtained during HLA typing is the most multivariate. The today's typing techniques make it possible to acquire information about the following:

– nucleotide sequences encoding the most significant regions of the antigen-recognition domains of MHC proteins;

– complete nucleotide sequences encoding the antigen-recognition domains;

– complete (excluding synonymous variants) nucleotide sequences of the exons encoding the entire structure of MHC proteins;

– complete (including synonymous variants) nucleotide sequences of the exons encoding the entire structure of MHC proteins;

– complete (including synonymous variants) nucleotide sequences of the exons encoding the entire structure of MHC proteins and complete nucleotide sequences of introns in the MHC genes;

– complete (including synonymous variants) nucleotide sequences of the exons encoding the entire structure of MHC proteins and complete nucleotide sequences of introns in the MHC genes, along with information about the expression levels.

The available data on the ongoing research developments aimed at combining the data on the nucleotide sequences of HLA genes with different integrity levels (resolution) and the structure of international HLA nomenclature are indirect evidence of the fact that there are currently typing data of almost all the above resolution types in the databases of world's registries [27, 28]. The typing data with different resolution are sometimes found within the same registry. An example could be the international DKMS registry (Germany, UK, Chile, Poland, South Africa, USA), where, despite the 6-year experience of typing performed based on six conventional HLA genes, in 2019 the German data set was still characterized by the presence of 100,000 donors typed by two HLA genes only [29].

In the Italian registry of bone marrow donors (as for 2017), there is a practice of using the so-called "primary requests" when performing the search for donors, in which the fact of match is determined based on the low-resolution HLA typing data. This procedure was recognized as useful in terms of accelerating the search for matched donors [30].

The NMDP procedures provide for the possibility of limiting to the first two of the completeness levels of information about the MHC genes presented in the above list at the time of entering the registry, however, at the time of activation it is necessary to perform typing aimed at determining the complete nucleotide sequences of exons (points 3–4), which corresponds to the term "high resolution" used in foreign procedures [31]. The list of the typed major histocompatibility complex protein molecules is also multivariate. For example, since the NMDP creation, the initial requirements specified only the HLA-A, HLA-B, and DR receptors as mandatory. By 2005, the requirement of additional HLA-C typing was added, and therefore the term 8/8 MUD (Matched Unrelated Donor) indicating the appropriate match standard came into practical use. The convincing data on the importance of such receptors as DP, DQ started to emerge over time, and the 10/10 MUD was gradually introduced into practice [32].

The designated areas of procedures and requirements belong to the category of typing outputs, while the technological process of acquiring those is also multivariate. Currently, several main types of technologies that can be used for HLA typing are distinguished: PCR-SSP (polymerase chain reaction with sequence-specific primers), PCR-SSOP (polymerase chain reaction with sequence-specific oligonucleotides probes), SBT (sequencing-based typing), next-generation SBT [33–37]. Furthermore, the last technologies from this list enable acquisition of the most complete genotyping results, while the PCR-SSP and PCR-SSOP techniques confine the resulting dataset to information about the sequences of antigen-binding regions; these are often linked to the catalogues of the abundant and well documented CWD alleles. The latter are designed to partially compensate the lack of information about the nucleotide structure of HLA macromolecules (insufficient resolution) and are used as the templates for targeted search when conducting PCR [38].

Today, thanks to the efforts of such organizations, as the American Society for Histocompatibility and Immunogenetics (ASHI), European Federation for Immunogenetics (EFI CWD), and China Marrow Donor Program, the CWD catalogues are maintained in the USA, European Union, and China [37–39].

Specific aspects of the Western BM and HSC registry functioning. Ensuring national security

All the organizational and methodological aspects of the work of world's registries of bone marrow and HSC donors considered so far can be classified as common. The reason is that these are realized within the framework of routine functioning and are not exclusive. However, the literature data suggest that the so-called specific aspects of the registry functioning (procedures and programs of functioning in emergency situations, such as disasters of all kinds) are also subjected to methodological workout. In 2012, the guidelines for international members of the organization on the implementation of the plan for countering natural, industrial or other man-made emergencies was issued under the aegis of WMDA [40].

The document outlines the range of main directions for organizing counteraction to the negative consequences of an emergency that has already occurred or is unfolding. It is noteworthy that the first paragraph is of general nature and implies the registry system response to the destructive processes and events that are not directly related to the registry function impairment. Given that such definition opens the door for the extremely broad interpretation, the assumption about the document authors' effort to lay foundation for the development of procedures for providing large-scale assistance to the population using the registry resources seems to be logical enough. The situation of mass radiation exposure to the doses of 5–10 Gy, when bone marrow transplantation is a life saving-procedure, represents an example of the increased demand for the donor potential of the bone marrow registries in the context of exposure to adverse factors and disasters. The strategic plan described in the document, in which the first paragraph shows the importance of forming the system for priority workout of the rating of potential requests for transplantation in crisis conditions, represents the potential additional evidence suggesting the possibility of working out the scenarios for countering radiation damage. Theoretically, prioritization of this kind can be very important under conditions of mass radiation exposure due to inability to simultaneously satisfy many potential recipients and the need to make tough decisions about the involvement of donor resources in one or another area.

Furthermore, it is necessary to mention that such an aspect directly related to registries, as the possibility of creating the BM and HSC banks also intended for autotransplantation, was discussed in the media with regard to the Fukushima nuclear accident. According to the report issued by Scienceinsider, the group of Japanese medical experts, including Tetsuya Tanimoto being a representative of the Japanese Cancer Association, addressed a letter to The Lancet journal on April 15, 2011, in which it expressed the need to organize a HSC bank for the plant employees [41]. The authors believe that this measure is intended to reduce negative effects of possible exposure to high radiation doses. To confirm the authors' opinion, Scienceinsider cites the words of Nelson Chao (Duke University in Durham, North Carolina), the expert in transplantation, who declares undoubted benefits of such measures for overcoming the effects of the radiation exposure associated with cancer treatment.

Some interesting facts related to the very moment of founding the US national registry of bone marrow donors are indirect evidence of the global registries' adaptation to the scenarios of nuclear disasters. Actually, in 1984, Al Gore, the congressman (who later became the US vice president) failed to overcome the resistance of the White House considering this initiative as untimely, despite huge personal enthusiasm and extensive political power. Support could not be achieved even during a special coordination meeting organized under the auspices of the NIH, and the concept of the registry was realized only in 1986 due to the fact that the US Navy was granted 1.2 million dollars. It is interesting that Captain Robert J. Hartzmann, head of the naval transplantation registry, was aware of all the nuances of financial arrangements [42]. Given the strategic importance of using nuclear power plants on the war ships constituting the basis of the US Navy striking force, this fact can show that there are specific reasons for creation of the world's largest BM and HSC registry, including that related to overcoming the effects of the personnel radiation exposure.

CONCLUSION

Thus, the review discusses the literature data on the long-term worldwide experience of using the bone marrow and HSC registries focused on the common and specific organizational and methodological aspects of the registry functioning. These data have been critically reviewed; the data credibility and practical value are beyond doubt.

In particular, in the RF, information about the citizens' high responsiveness to the bone marrow donating popularization programs, the features of arranging recruitment, the requirements for the registry population considering the multinationality and heterogeneity of ethnic composition, and the algorithms of it`s statistical approximation are of great interest in terms of implementation of such information systems. In the context of technological support, the data on the diversity of algorithms to search for matched donors, including those targeted to the using of the alternative transplantation material sources, such as Cord Blood Unit и Peripheral Blood Stem Cells, attract attention, along with the fact of the global registries' adaptation to the diverse HLA typing methods. The data on the potential relationship between the BM and HSC registries and the problem of ensuring national security, are of special importance, including in the context of protecting the population against the effects of the disasters, emergencies, and terrorist attacks associated with the development of bone marrow syndrome in victims. The fact of scientific arguments in favor of creating the BM and HSC biobanks for populations at high risk in terms of the radiation exposure factor deserves special mention in this regard.

The relevance and practical significance of the data provided in the review are confirmed by the fact of underrepresentation of the themes related to the activities of the national registries of bone marrow donors, specifically the Federal Registry of Bone Marrow and HSC Donors, in the Russian scientific literature. Currently, such papers are focused mainly on the legal aspects of the registries' activity [43, 44], reiteration of the need to it`s creation [45], and practical results of the work of only one registry, the Rosplasma Center of FMBA of Russia [16].

Given the above, it is reasonable to put forward a hypothesis that further accumulation of the pool of domestic papers focused on the Russian experience in this sphere will make it possible to take full advantage of the foreign research data provided in the review as the basis for comparison and arrangement of the productive debate about the optimal ways to develop the Federal Registry of Bone Marrow and HSC Donors.

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ESSENTIAL TREMOR: MODERN VIEW OF THE PROBLEM AND NEW NEUROSURGICAL TREATMENT OPTIONS

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The review is focused on essential tremor (ET), the most common extrapyramidal system disorder. Current understanding of the disease pathogenesis is provided; issues of classification and differential diagnosis are discussed. Modern ET treatment methods include therapeutic approaches and surgical interventions. The benefits of the new ET treatment method, the magnetic resonance-guided focused ultrasound treatment (MRgFUS), are described; the world's experience of using the method, indications and contraindications are summarized.

Keywords: essential tremor, treatment, diagnosis, thalamotomy, MRgFUS

Author contributions: Katunina EA — manuscript writing; Gryaznev RA — literature review, data acquisition and processing, manuscript editing; Shipilova NN, Katunin DA, Malykhina EA — editing, formatting, approval of the final version of the article.

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ЭССЕНЦИАЛЬНЫЙ ТРЕМОР: СОВРЕМЕННЫЙ ВЗГЛЯД НА ПРОБЛЕМУ И НОВЫЕ ВОЗМОЖНОСТИ НЕЙРОХИРУРГИЧЕСКИХ МЕТОДОВ ЛЕЧЕНИЯ

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Обзор посвящен наиболее часто встречающемуся заболеванию экстрапирамидной системы — эссенциальному тремору (ЭТ). Приведены современные представления о патогенезе заболевания, затронуты вопросы классификации и дифференциального диагноза. Современные методы лечения ЭТ включают терапевтические подходы и хирургические вмешательства. Описаны преимущества нового перспективного метода лечения ЭТ — терапия фокусированным ультразвуком под контролем МРТ (ФУЗ-МРТ), обобщен имеющийся на сегодняшний день мировой опыт его применения, показания и противопоказания.

Ключевые слова: эссенциальный тремор, лечение, диагностика, таламотомия, ФУЗ-МРТ

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Essential tremor (ET) is the most common extrapyramidal system disorder. According to the data of meta-analysis, the average prevalence is 0.9%; it increases with age and reaches 21.7% in the group of patients over the age of 95 years [1]. The ideas about ER have changed considerably in the recent years. This is true for the issues of etiology and pathogenesis, as well as for clinical aspects. ET has been considered as an inherited monosymptomatic disorder associated with the local subcortical structural damage for a long time. To date, the range of ET symptoms has expanded considerably to include both motor and non-motor manifestations. The aim of the review was to summarize the literature data on modern ideas about pathomorphology of the disease as a progressive neurodegenerative process, clinical aspects and treatment methods.

Ethiology and pathogenesis

The term "essential tremor" was first introduced into clinical practice in 1874 by Pietro Burresi, who described the 18-year-old patient with severe action tremor. ET had been regarded as "benign tremor" for a long time. However, today, it is clear that this disorder can have serious functional and psychological consequences: 15% of affected individuals have severe disability; in 80% of patients, tremor significantly limits their daily activity by disturbing the eating process, writing, and execution of target-directed movements [2].

According to the definition issued by the International Parkinson and Movement Disorder Society (MDS) in 2018, ET is an action tremor in the hands with the disease duration of at least 3 years that can be combined with tremor of other localization (for example, head, voice or lower limb tremor) in the absence of other neurological signs, such as dystonia, ataxia or parkinsonism [3].

Based on the etiological principle of distinguishing tremor types, ET can be genetically defined, sporadic or familial (Figure).

ET is commonly inherited by autosomal dominant transmission with incomplete penetrance and had variable in 40–70% of patients [4]. The anticipation phenomenon with the earlier onset and more severe clinical manifestations in subsequent generations is observed in a number of families [5]. The ET familial variants can develop at any age, including at the age over 60 years, however, early onset is more common. According to the data of some studies, familial forms have two age peaks of the disorder onset: early age (including childhood) and older age. No clear incidence peaks are observed among individuals with sporadic forms [6]. The age of onset variability observed in individuals with familial can be

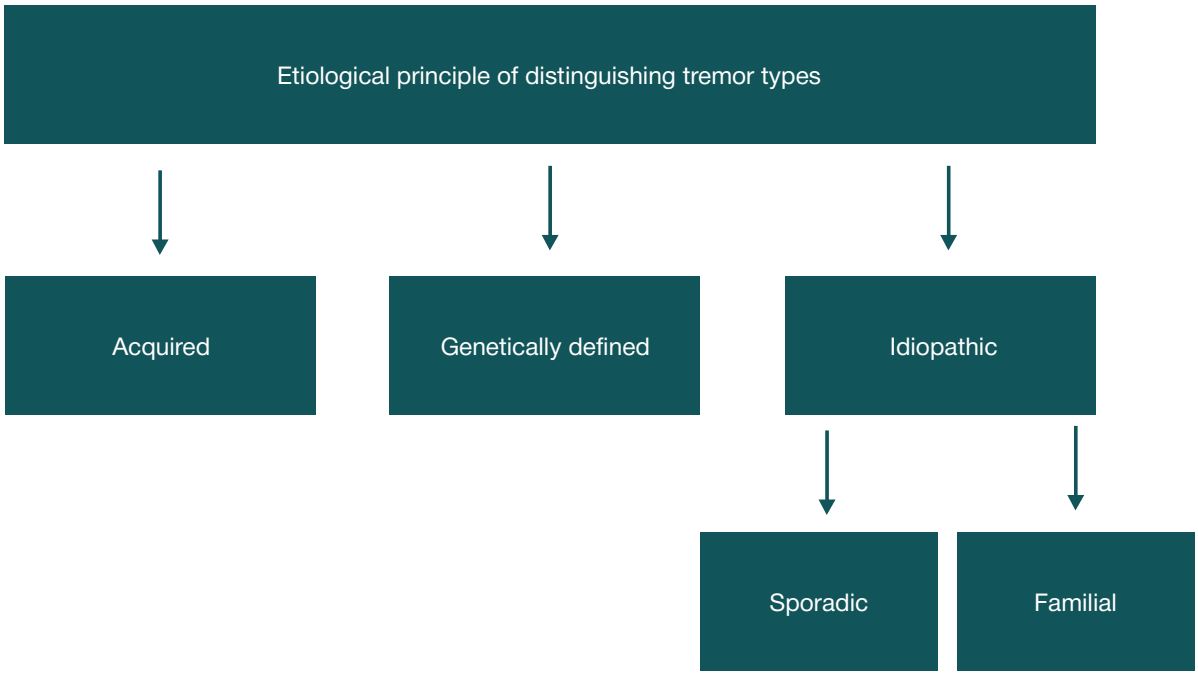

Figure. Consensus classification of tremor compiled by the International Movement Disorder Society in 2018 based on the etiological principle

associated with the diversity of genetic risk factors and the effects of environmental factors on the expression of certain genes [6].

Despite apparent genetic predisposition, the gene responsible for the development of ET has not yet been identified. The whole genome sequencing has shown that ET is associated with polymorphism of the LINGO1 gene playing an important role in the mechanisms underlying neuroplasticity. The protein associated with this gene is involved in axonal regeneration and cell differentiation [7]. It has been shown that the LINGO-1 expression increase following the neuronal damage is associated with demyelination of axons. The decrease in the LINGO-1 activity can contribute to the recovery of neurological deficit with increasing survival rate of neurons and dendritic growth [8].

In recent years, there is growing evidence that ET is a neurodegenerative disorder. The most prominent alterations are found in the cerebellum and inferior portions of the brainstem. Progressive Purkinje cell death, degenerative changes affecting the dentate nucleus, white matter atrophy are observed in the cerebellum. The axonal “torpedoes” that represent proximal axonal swellings on Purkinje cells and contain altered neurofilaments are a common nonspecific pathomorphological marker of ET [9]. Furthermore, Lewy bodies are found in the brainstem of individuals with ET, primarily in the locus coeruleus, the axons of which form synapses with the cerebellar Purkinje cells [9]. Perhaps, damage to the locus coeruleus cells reduces the stimulating noradrenergic effect on the Purkinje cells and leads to the secondary Purkinje cell dysfunction. Thus, the results of morphological studies together with the functional neuroimaging data suggest the important role of the cerebellum in the ET pathogenesis [10]. At the same time, primacy of the tremorogenic role played by the cerebellum is still a matter of debate. There is an opinion that abnormal activity of the cerebellar structures can be secondary to the pacemaker activity of the inferior olivary or thalamic nuclei capable of generating spontaneous bursting oscillation [11]. Abnormal activity of the inferior olivary and/or cerebellar nuclei is transmitted to the motor cortical areas through the dentato-rubro-thalamic tract and realized in the form of alternating or synchronous contraction of the agonist and antagonist muscles manifested by tremors [12]. The thalamic nuclei (primarily the Vim nucleus) play a role of a kind of the relay station between the subcortical structures and the motor cortex. That is why the Vim nucleus is the most common target for tremor neurosurgery (deep brain stimulation, magnetic resonance-guided focused ultrasound treatment (MRgFUS), radiofrequency ablation).

Purkinje cells, Golgi cells, dentate nuclei, cerebellar basket cells are GABAergic neurons having the inhibitory function. The role of GABAergic systems in the ET development has been confirmed by the efficacy of such drugs, as primidone, gabapentin, pregabalin, topiramate, benzodiazepines, targeting the GABA receptors. The well known beneficial effect of alcohol in individuals with ET can be also explained by the indirect agonistic effect on the GABA receptors. Its effect on tremor is likely to be realized through reduction of aberrant synchronization of the inferior olives. The antagonistic effect on the low-voltage-activated calcium channels resulting in the increase in the T-type current in the inferior olive can represent one more mechanism underlying the effect of ethanol in ET [13].

Peripheral mechanisms are also likely to be involved in tremor mechanisms. The effect of nonselective beta-blockers used as the first-line drugs for treatment of ET is realized due to the effect on the skeletal muscle β_2-receptors found in the muscle spindles [14].

Clinical features and diagnostic criteria

The clinical features of the disorder include postural tremor (tremor of outstretched hands) and kinetic tremor. Tremor usually develops symmetrically and predominates in the distal limbs. In a number of cases, the asymmetric onset is possible, when the second hand is involved within months. The tremor frequency is stable; it is in the range of 4–12 Hz. The amplitude is very variable, it shows daily fluctuations and depends on the patient’s emotional state and fatigue severity. In contrast to Parkinson's disease (PD), the feature of the ET-associated postural tremor is that it occurs immediately after outstretching the hands, with no delay typical for PD. Kinetic tremor tends to increase in the terminal point of targeted

Table. Diagnostic criteria for ET, ET plus and exclusion criteria for ET, ET plus issued by the International Parkinson and Movement Disorder Society (2018)

Criteria for ET	Criteria for ET plus	Exclusion criteria for ET and ET plus
1. Isolated bilateral kinetic hand tremor 2. Persisting for at least 3 years 3. Tremor can be combined with tremor of other localization (for example, in the head, vocal cords, lower limbs) 4. No other neurological symptoms, such as dystonia, ataxia, parkinsonism)	1. Tremor compliant with the ET characteristics in the presence of additional neurological symptoms of undefined clinical significance: tandem gait impairment, disguised dystonic attitude, memory impairment or other mild neurological symptoms that are not enough to diagnose an additional syndrome or disorder 2. Essential tremor with added resting tremor	1. Isolated focal tremor (head, voice) 2. Orthostatic tremor with the frequency exceeding 12 Hz 3. Tremor associated with execution of certain tasks or manifesting itself in a certain position 4. Sudden onset, stepwise progression

movement (terminal tremor). Hand tremor can involve proximal parts and go with tremor in the head ("yes-yes" or "no-no"), chin, vocal cords, trunk, lower limbs. The isolated head/voice tremor that was earlier considered as an ET variant is currently a reason for exclusion of the diagnosis considered to be a manifestation of focal dystonia. According to the international criteria, individuals with ET should have no other neurological symptoms (parkinsonism, ataxia, dystonia). In case of postural tremor combined with bradykinesia, one should first think about PD.

The disorder tends to progress, which confirms its neurodegenerative nature. However, the ET progression rate is highly individualized. In some patients, tremor persisting for many years does not disturb their daily activity. In other patients, it results in the reduced working capacity, capability of writing and working on the computer, disturbed eating and self-care at home. Some patients show not only growing tremor severity and involvement of other parts of the trunk, but also accession of other symptoms, such as parkinsonism (rigidity, resting tremor, mild hypokinesia), cerebellar disorders (mild intention tremor, dysmetria, impaired tandem gait), dystonia of various localization. These are usually minor "mild" symptoms. According to the international criteria, ET plus is diagnosed in such cases [3] (Table). Moreover, in some patients, such symptoms can be identified during the first years of the disease.

The epidemiological research conducted shows that ET plus is more common than "pure" ET, especially among individuals with the late-onset disease [15]. In this regard, there is an ongoing debate, whether ET plus is a separate disease entity, separate syndrome or it represents the later-stage ET, in the group of experts [16, 17]. The lack of pathomorphological differences from ET is the most significant argument in favor of the fact that ET plus is not a separate disease entity [18]. Interpretation of ET plus as a separate syndrome that is a possible transitional form between ET and such disease entities, as PD, spinocerebellar ataxia, different variants of dystonia, seems to be more potent [3, 16, 17, 19]. The cases of postural tremor combined with resting tremor are the most challenging in terms of differential diagnosis between ET plus and PD. In contrast to PD, the resting tremor associated with ET plus has the same frequency as the postural-kinetic tremor and, which is more important, there is no bradykinesia and rigidity.

Positive response to low doses of alcohol as a diagnostic sign was excluded from the list of international criteria for ET (2018) as a non-specific and non-permanent symptom, however, the 3-year follow-up period remains in the list [3]. Such a time period is essential to reduce the likelihood of the erroneous diagnosis of other neurological syndrome (for example, dystonia, parkinsonism or ataxia). The term "indeterminate tremor" can be found in the literature, which is used in cases of shorter disease duration [20].

It was believed that ET was a monosymptomatic disease in which there were no non-motor symptoms for a long time. At the same time, a large body of research has emerged showing the development of cognitive impairment, anxiety disorders, and depression in individuals with ET, which further enhances the disease heterogeneity. The cognitive impairment profile is considered to be associated with dysfunction of the frontostriatal or cerebellar thalamocortical systems. The risk of cognitive impairment increases with age. One of the studies focused on assessing the relationship between the ET age of onset and the cognitive impairment has shown that 70% of patients diagnosed with ET had a cognitive deficit at the age of 65 years [21]. Given the neurodegenerative nature of ET and the axonal dysfunction detected, we can assume the possibility of the process expansion from the spinocerebellar structures to the cortical areas.

There are data on higher prevalence of anxiety and depression among patients with ET compared to the group of healthy individuals [22]. The lack of correlation between the tremor severity and the severity of anxiety disorder probably suggests that the anxious personality profile can be a primary disease manifestation, not the consequence of the psychological disorder caused by severe disabling tremor.

The drugs that are currently used to treat ET are symptomatic. These were developed and approved for other indications. Despite numerous attempts to develop new medicinal products, primidone and propranolol remain the first-line drugs for treatment of ET [23]. The drugs are almost equally effective. According to the expert panel of the American Academy of Neurology (AAN), the grade of evidence for their effect is defined as A [24]. According to the research, the use of these drugs does not result in significant reduction of tremor in about 50% of patients [25]. Moreover, in cases of long-term course of the disease, it is almost never possible to achieve complete tremor relief. In an effort to treat tremor, one has to use high doses of drugs, their combinations, thereby increasing the risk of side effects, such as drowsiness, weakness, poor concentration, bradycardia, hypotension, and reducing adherence to treatment. As a result, many patients use subtherapeutic doses or prefer to completely stop taking the drug. The effective dose of propranolol is in the range of 60–120 mg per day. However, there is a need to increase the dose to 240–360 mg/day in a number of patients [26]. It is necessary to monitor bradycardia and arterial hypotension in all dose ranges in order to prevent drug-induced syncope. Furthermore, propranolol should be prescribed with caution to individuals with obstructive lung diseases, bronchial asthma. The effective dose of primidone is in the range of 150–750 mg/day, and the average effective dose is 300 mg/day. Potential side effects include vertigo, instability, drowsiness, fatigue. The combination of propranolol and primidone has a more prominent therapeutic effect [27].

The second-line drugs include topiramate, alprazolam, gabapentin, clonazepam (grade of evidence B). Topiramate is the only drug that has passed a randomized placebo controlled clinical trial [26]. The average effective dose of topiramate varies between 215 and 333 mg/day. Possible side effects include paresthesia, concentration problems, weight loss, nausea,

insomnia, depression. Topiramate should be prescribed with caution to patients with impaired renal and liver function.

The recommended dose of alprazolam is 0.75–1.5 mg/day. In the reviews by AAN and the International Movement Disorder Society (MDS) alprazolam is considered as "probably effective and possibly beneficial" when used for treatment of tremor. The side effects of alprazolam can be represented by drowsiness, vertigo, ataxia, potential addiction. It is more often recommended to use alprazolam from time to time as a supplementary drug.

The risk of addiction syndrome is also typical for clonazepam. Clonazepam is usually recommended for severe head tremor or dystonic tremor. Despite the fact that AAN recognizes gabapentin as a "probably effective" drug [28], and a placebo controlled trial has been conducted showing that gabapentin in a dose of 1200 mg/day is effective against tremor, the drug is rarely used, mostly in combination with other medications, when the first/second-line drugs are ineffective.

It should be noted that the ET treatment goal is not to completely relieve tremor, but to minimize the tremor-associated functional limitations, reduce social maladjustment.

Surgical treatment

In case of tremor refractory to medication, the patient can be referred to surgery [23, 25]. Today, surgical treatment includes deep brain stimulation (DBS), radiofrequency ablation, Gamma Knife, MRgFUS. The advantages of DBS include the possibility of simultaneous bilateral stimulation, adjustment of the stimulation parameters depending on the patient's response or side effects. Prolonged high frequency electrical stimulation, the key "relay" structure (Vim nucleus of the thalamus) is exposed to, functions as an "artificial pacemaker" in the brain, imposing the artificial pattern of neuronal discharges on the stimulated nucleus and thereby ensuring desynchronization of abnormal rhythm in the sensorimotor circles. The DBS efficacy in ET reaches 80% [29]. However, the invasive nature of the procedure creates the risk of hemorrhage and infectious complications reported in about 5–7% of patients [30]. Side effects can be also associated with electrical stimulation itself and be manifested by dysarthria, gait instability, paresthesia. The risk of dysarthria and ataxia increases when bilateral stimulation is applied. Furthermore, the effectiveness of tremor control decreases within a few years, which can be due to the lead migration, developing tolerance to electrical stimulation or the disease progression [31]. The issue of adverse DBS effects on the cognitive function is discussed, along with the increased risk of anxiety disorders and depression [29–31]. There are many contraindications for DBS related to focal brain atrophy, taking anticoagulant drugs, and age limits, which require thorough selection of patients. The patients, who have undergone such surgery, should sometimes contact medical institutions for stimulation mode adjustment and electrical stimulator replacement after 4–5 years.

Radiofrequency ablation is an invasive method (an electrode is introduced into the target point). Despite a significant immediate effect on tremor (reduction by 56.4–90%), it is associated with the risk of intracerebral hemorrhage, developing hemiparesis, dysarthria, ataxia, cognitive decline [32].

The Gamma Knife thalamotomy involves the use of ionizing radiation, despite its non-invasive nature and the fact that it is conducted without anesthesia. The main shortcoming of the method is the lack of intraoperative clinical assessment, along with the unpredictable lesion size and timing of both therapeutic effect (4–8 months on average) and complications [33].

In 2016, the US Food and Drug Administration (FDA) approved a new ET surgical treatment method: destruction of subcortical structures using MRgFUS. In 2017, this treatment method was approved by the Federal Service for Surveillance in Healthcare (Roszdravnadzor) in the Russian Federation. In 2019, the MRgFUS method was included in the evidence-based review of ET treatment methods published by MDS [26]. The MRgFUS advantages over other surgical treatment methods include its non-invasive nature, immediate effect, accuracy of impact on the selected target, and the possibility of monitoring the thermal exposure due to continuous MRI control applied when performing surgery. No implanted devices in the patient, no ionizing radiation, and no need for repeated visits to medical institutions aimed at adjusting the devices are the important features of MRgFUS [34].

The Vim nucleus of the thalamus is the main target for ET treatment using MRgFUS. The effect on the stereotactically verified target point is ensured by initial heating of the selected area (2 mm in diameter) to 40–45 °C with the focused ultrasound through the skull bones (multiple serial short sonications from 1024 sources (650 kHz) placed around the head). Further temperature increase in the target zone causes the ablation damage. The constant visual and verbal contact with the patient is maintained during the procedure in order to estimate the tremor changes upon exposure and record the fact of any adverse effect (for example, paresthesia, dysarthria) after each sonication. Parameters of each subsequent sonication can be adapted to the clinical response during surgery. The temperature is increased and the ultrasound exposure is used until the tremor suppression is achieved. The emergence of paresthesia or any other adverse effect constitutes grounds for the target localization adjustment [35].

The skull is the main obstacle for the ultrasonic wave on its way to the target point, since the bone can both reflect and absorb ultrasonic waves, hampering the acoustic energy transmission and attenuating the MRgFUS therapeutic effect. The skull bone consists of two compact plates (outer and inner) separated by a layer of spongy bone (trabecular or diploe). The coefficient estimating the resolving power of the ultrasonic waves propagating through the skull bone is referred to as Skull Density Ratio (SDR). This parameter calculated based on the head CT scans represent a median ratio between the spongy bone and compact layer of the skull bone in the target area.

The first open-label trial involving the use of MRgFUS in ET was conducted in 2013. The authors of the study reported a significant reduction of tremor and improvement of the quality of life, along with the low number of reversible side effects (paresthesia, dysarthria, ataxia) [36]. In 2016, the results of the blind multicenter randomized trial of parallel groups with the mock surgery used as a control were published. The study involved 76 patients with moderate-to-severe refractory tremor. The tremor severity decreased by 47% within 3 months after unilateral thalamotomy showing significant differences from the mock surgery group ($p < 0.001$). Tremor reduction was accompanied by the daily functioning and quality of life improvement 3 months after treatment. The researchers reported the decrease in tremor severity by 40% relative to baseline and the decrease in maladjustment by 62% [37]. High productivity of the research conducted allowed FDA to approve MRgFUS for treatment of ET. According to the currently available research results, the tremor reduction range is 40–90% [34, 38]. The persistent positive effect on tremor was demonstrated in the 4-year open-label and 5-year retrospective studies [39, 40]. In these studies, paresthesia and numbness, gait instability and mild contralateral muscle weakness that tended to regress by month 3 after surgery were the most frequent adverse events occurring within the first weeks after the MRgFUS thalamotomy. Extinction of postoperative

neurological symptoms is considered to be associated with the reduction and gradual disappearance of perifocal edema.

In 2018, meta-analysis of the outcomes and complications of the MRgFUS thalamotomy used for treatment of ET was conducted. A total of nine studies involving 160 patients published in 2013–2018 were included. Vertigo was noted as the most common intraoperative complication that occurred in 43.4% of cases, it was followed by nausea and vomiting (26.85%). Ataxia (32.8%) and paresthesia (25.1%) were most often detected 3 months after thalamotomy. Regression of ataxia was observed in the majority of patients 12 months after surgery, and paresthesia became the most common permanent complication (15.3%) [41]. According to the results of other studies, no residual side effects were observed by the end of the 4-year follow-up period [42], gait disorders or numbness emerged in 11% of patients by year 5 of follow-up [39].

It should be noted that MRgFUS has no adverse effect on cognitive functions [43]. The recently published meta-analysis provides assessment of the effect of thalamotomy performed using radiofrequency ablation, Gamma Knife, and MRgFUS on cognitive functions. In general, the unilateral thalamotomy safety is emphasized in the review. There was adverse effect on verbal fluency only: the decrease in the number of phonemic associations was mainly observed. The separate sub-analysis performed in the MRgFUS group revealed no cognitive function alteration in any of the areas [44].

The first attempts are made to perform bilateral thalamotomy in order to treat ET using MRgFUS. Thus, in 2014, outcomes of the staged thalamotomy were published. The interval between surgical procedures was 12 months or longer. The tremor severity decreased by 60% after the second surgery; the side effects occurred in 2.6% of patients [45]. Safety of the staged bilateral thalamotomy comparable with that of unilateral surgery was demonstrated in the BEST-FUS trial [46]. Possible adverse events associated with bilateral surgery include transient gait instability, dysarthria, dysphagia, perioral numbness, hemihypesthesia, taste disturbance. The adverse events are mild-to-moderate and regress within 3–6 months [47, 48].

The Vim nucleus of the thalamus is the target of bilateral surgery performed using MRgFUS. However, the papers have emerged reporting the use of the cerebellothalamic tract as a target point for treatment of ET [34], which reduces the risk of side effects. The 12-month prospective study has demonstrated the decrease in hand tremor by 93% and maladjustment by 51% [49].

The MRgFUS method should be recommended to patients with severe disabling tremor that disturbs their daily activity and is resistant to medication. Multiple focal alterations in the brain, preceding brain intervention (DBS, destructive stereotactic surgery, electroconvulsive therapy), high risk of hemorrhage, contraindications to high-field MRI, claustrophobia, mental disorders, severe cognitive impairment, decompensated somatic disorder, SDR $\geq 0.4 \pm 0.05$ (based on the CT data) are contraindications to surgery. It should be noted that density of the skull is a relative contraindication. In the last two years, the papers have emerged that suggest technical feasibility, safety and efficacy of MRgFUS in patients with low skull density (≤ 0.4). Limitation of the number of sonications, adjustment of the maximum impact energy and maximum temperature ensure persistent therapeutic effect [50].

CONCLUSION

ET is among the most common neurological disorders that can be faced by physicians of various specialties. Despite the long history of studying the disorder, many issues of etiopathogenesis are poorly understood and require more extensive epidemiological research with pathomorphological confirmation. The currently available diagnostic criteria for ET are clinical and require monitoring the patient for a certain period, which is sometimes associated with problems in daily clinical practice. In the recent years, the capabilities of neurosurgical treatment have expanded, along with the use of symptomatic agents for treatment of tremor. The results of using such minimally invasive method, as MRgFUS, characterized by high efficacy and safety, are the most encouraging. However, the global and domestic experience of using this technique is only accumulating.

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IN VITRO ASSESSMENT OF IMMUNOGENICITY IN CHONDROCYTES OBTAINED FROM THE B2M KNOCKOUT INDUCED PLURIPOTENT STEM CELLS

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Today, the cell-based technologies are one of the instruments used for the cartilage tissue repair. Creation of a universal hypoimmunogenic cartilage tissue graft from the differentiated derivatives of induced pluripotent stem cells (iPSCs) might solve the problem of the lack of the cartilage cell product. However, currently there is little data on immunogenicity of such tissue-engineered preparations. The study was aimed to create a cartilage implant from the differentiated derivatives of the B2M-deficient iPSCs and assess its immunogenicity. The previously developed protocol was used to ensure differentiation of both wild-type and B2M knockout iPSCs into chondrocyte-like cells. After quality control of the resulting cell lines by conducting polymerase chain reaction and immunocytochemical assessment, the resulting cell lines were co-cultured with the peripheral blood mononuclear cells of a healthy donor. When co-cultivation was over, activation and degranulation of $CD8^+$ T cells was assessed by flow cytometry analysis based on the CD69 and CD107a expression on the cell surface, respectively. The iPSC-derived chondrocytes expressed the cartilage tissue markers. Flow cytometry analysis revealed no substantial differences in immunogenicity between the derivatives of wild-type and B2M knockout iPSCs, as well as from the cartilage tissue cells of a healthy donor. Immunogenicity of chondrocyte-like cells was higher than that of hypoimmunogenic non-edited iPSCs. The B2M knockout iPSCs demonstrated a trend towards greater activation of $CD8^+$ T cells. Thus, the B2M knockout in the iPSC-derived chondrocytes had no significant effect on the tissue immunogenicity. It is necessary to further edit the genes encoding MHC II and CD47 to obtain a less immunogenic product.

Keywords: iPSCs, regenerative medicine, chondrogenesis, chondrocytes, immunogenicity

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IN VITRO ОЦЕНКА ИММУНОГЕННОСТИ ХОНДРОЦИТОВ, ПОЛУЧЕННЫХ ИЗ ИНДУЦИРОВАННЫХ ПЛЮРИПОТЕНТНЫХ СТВОЛОВЫХ КЛЕТОК С НОКАУТОМ B2M

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В настоящее время клеточные технологии являются одним из инструментов по восстановлению хрящевой ткани. Создание универсального гипоиммуногенного трансплантата хрящевой ткани из дифференцированных производных индуцированных плюрипотентных стволовых клеток (ИПСК) могло бы решить проблему нехватки хрящевого клеточного продукта. Однако на сегодняшний день мало данных об иммуногенности таких тканеинженерных препаратов. Целью работы было создать хрящевой имплант из дифференцированных производных ИПСК, дефицитных по B2M, и оценить его иммуногенность. С помощью ранее разработанного протокола дифференцировали ИПСК как дикого типа, так и с нокаутом B2M в хондроцитарные производные. После проверки качества полученных линий методом полимеразной цепной реакции и иммуноцитохимическим исследованием кокультивировали полученные линии с мононуклеарными клетками периферической крови здорового донора. По окончании кокультивации методом проточной цитометрии оценивали активацию и дегрануляцию $CD8^+$-Т-лимфоцитов по экспрессии CD69 и CD107a на поверхности клеток соответственно. Хондроцитарные производные ИПСК экспрессировали маркеры хрящевой ткани. Цитометрический анализ не выявил существенных различий между иммуногенностью хондроцитарных производных ИПСК с нокаутом и без нокаута B2M, а также клетками хрящевой ткани здорового донора. Иммуногенность хондроцитарных производных была выше, чем у гипоиммуногенных нередактированных ИПСК. Нокаутированные по B2M ИПСК демонстрировали тенденцию к большей активации $CD8^+$-Т-лимфоцитов. Таким образом, нокаут B2M в хондроцитарных производных ИПСК не оказал существенного влияния на иммуногенность ткани. Необходимо дополнительное редактирование генов, кодирующих MHC II и CD47, для получения менее иммуногенного продукта.

Ключевые слова: ИПСК, регенеративная медицина, хондрогенез, хондроциты, иммуногенность

Финансирование: получение хондроцитарных производных из ИПСК выполнено в рамках государственного задания №122032300191-2 «Органоид-22». Иммуноцитохимический и ПЦР-анализы экспрессии хондрогенных маркеров в хондроцитарных проиводных ИПСК, а также оценка иммуногенности этих хондроцитарных производных проводились в рамках проекта РНФ #22-15-00250 «Сравнение хондрогенного потенциала хрящевой ткани, полученной с помощью первичных культур хондроцитов и дифференцированных производных индуцированных плюрипотентных стволовых клеток».

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Restoration of cartilage tissue using the cell-based and tissue-engineered products is an urgent medical task due to high prevalence of inflammatory degenerative diseases involving the surface layers of bone and cartilage tissues. Gonarthrosis, the cartilage tissue disorder, is most prevalent all over the world. This multifactorial degenerative dystrophic disease is characterized by involvement of the articular cartilage, subchondral and metaphyseal bone, as well as the synovial membrane, ligaments, capsule, and muscles, and is associated with the emergence of osteochondral masses manifesting themselves in the joints experiencing pain and a limited range of motion. The annual incidence of gonarthrosis in Russia is about 80,000 cases. According to the epidemiological research data, 8–20% of the adult population suffer from this disorder, depending on the region [1].

Gonarthrosis progression is observed year by year; different treatment approaches are used at different disease stages. According to domestic clinical guidelines, noninvasive therapy combining pharmacological and physiotherapeutic methods is recommended at early stages of the disease [2]. It should be noted that the pharmacological component of therapy involving the use of non-steroidal anti-inflammatory drugs (NSAIDs) and steroids (short course) is symptomatic and has almost no effect on the hyaline cartilage regeneration and functional activity. At the later disease stages, when noninvasive treatment is ineffective and the cartilage tissue damage is significant, there is a need for surgical intervention including abrasion chondroplasty, corrective osteotomy, and joint replacement [3].

The use of cell products for the damaged cartilage tissue repair has become a new direction of regenerative medicine. In such cases, mesenchymal stem cells (MSCs), autologous and allogeneic chondrocytes can be the sources of cells.

Despite the fact that the cell products based on autologous chondrocytes for cartilage tissue repair are already present in the medical and biotechnology market, such products have a number of limitations. First, so far these are aimed mainly at adjusting minor articular cartilage damage [4, 5]. Second, for a number of reasons, it is often impossible to obtain enough autologous chondrocytes to create a full-fledged cartilage implant [2]. Third, in individuals with some cartilage tissue disorders, autologous cell material yields chondrocytes with decreased function, which results in the need for long-term cell cultivation aimed at accumulating the required amount of material [6, 7]. In this regard, induced pluripotent stem cells (iPSCs) represent a promising and capacious source of chondrocyte-like cells, however, the data on their safety and capability of forming the full-fledged functional cartilage tissue are rather limited, while the data on immunogenicity are controversial [8, 9]. Moreover, obtaining iPSCs, their further differentiation, creation of the graft is a lengthy and expensive process that requires optimization and standardization. The literature contains information about successful engraftment of the iPSC-derived chondrocytes in the monkey articular cartilage. However, the cartilage tissue defect was small, therefore, further research involving other models is required. As for immunogenicity assessment, transplantation into cartilage defects caused no immune response in macaques, while the organoid transplanted into the osteochondral defects remained intact, but was surrounded by a large number of $CD3^+$ T cells [10].

Creation and the use of cartilage grafts based on allogenic iPSCs differentiated into chondrocytes showing decreased immunogenicity, which are universal for all recipients, seem to represent a promising approach. A number of studies demonstrate successful creation of hypoimmunogenic grafts based on the MHC knockout iPSCs [11, 12]. It has been shown that the allogenic cartilage graft obtained from the B2M knockout iPSCs contributes to accumulation of natural killers (NK cells) around the osteochondral defect *in vivo* [13]. However, such data on human iPSC-derived chondrocytes have yet to be obtained.

The study was aimed to compare immunogenicity of the samples of tissue-engineered cartilage preparations obtained *in vitro* from various cell sources.

METHODS

Cultivation of wild-type and B2M knockout iPSC lines

The iPSCs (hereinafter, iPS) were cultured as previously reported [14]. The B2M knockout iPS cell line (iPS_dB2M) was obtained earlier [15].

iPSC differentiation into chondrocyte-like cells

The thawed iPS and iPS_dB2M (used to obtain wild-type (iCh) and B2M knockout (iCh_dB2M) chondrocyte-like cells, respectively) were seeded into 6-well plates pre-treated with Matrigel (Corning; USA), in the mTeSR1 medium (STEMCELL Technologies; Canada). These were cultured in the CO_2 incubators at +37 °C and 5% CO_2 until a monolayer was formed. All further incubations were also carried out in the CO_2 incubators at +37 °C and 5% CO_2. The culture was transferred into the DMEM/F12 medium (Gibco; USA) supplemented with 10% FBS (HiMedia; India), 1% GlutaMAX (Gibco; USA), 1% penicillin/streptomycin (PanEco; Russia), 10 μM Chir (Miltenyi Biotec; USA), 10 nM retinoic acid (Miltenyi Biotec; USA). It was cultured for two days. Then the medium was replaced with DMEM/F12 with 10% FBS, 1% GlutaMax, 1% penicillin/streptomycin, 10 ng/mL TGFβ (Miltenyi Biotec; USA), 10 ng/mL BMP2 (Miltenyi Biotec; USA), B27 (Miltenyi Biotec; USA), 10 μM ascorbic acid (Sigma; USA), 1% insulin-transferrin-selenite (PanEco; Russia). This was cultured for two weeks. Then the cells were transferred to two wells on the 6-well plate. After differentiation, the cells were cultured in the DMEM/F12 medium supplemented with 10% FBS, 1% GlutaMax, 1% penicillin/streptomycin, 10 ng/mL TGFβ, 10 ng/mL BMP2.

Quality control of the iPSC-derived chondrocytes. Immunocytochemistry assessment

The monolayer cultures fixed in the 4% paraformaldehyde (PFA) were treated with the 0.1% Triton-X100 solution: for 20 min to ensure staining for a nuclear marker, for 10 min to ensure staining for surface and cytoplasmic markers. After permeabilization the cultures were treated with the blocking solution based on the 0.01M PBS with 3% goat serum and 0.1% Tween for 30 min.

The monolayer cultures were stained with primary antibodies against the Sox 9, nuclear marker of chondrogenesis (Rabbit, 1 : 400; Invitrogen, USA), aggrecan, the proteoglycan cartilage extracellular matrix marker (Mouse, 1 : 500; Invitrogen, USA), type II collagen, the marker of the fibrillar extracellular matrix of hyaline cartilage (Rabbit, 1 : 200; Abcam, UK), and type I collagen, the marker of fibrous cartilage (Rabbit, 1:800, Invitrogen, USA). Staining involving the use of primary antibody solutions based on the blocking solution was performed for 1.5 h at room temperature. Then the cultures were triple washed with 0.01 M PBS.

Alexa Fluor 555 (Goat, Anti-Rabbit, 1 : 500) and Alexa Fluor 546 (Goat, Anti-Human, 1:500) (Invitrogen; USA) were used for

Table. Primers used in the study

Gene	Primers used in the study 5'→3'	Product size, bp
SOX9	F: GAAGTCGGTGAAGAACGGGC R: CACGTCGCGGAAGTCGATAG	283
ACAN	F: AGGAGTCCCTGACCTGGTTT R: CCTGACAGATCTGCCTCTCC	167
COL1A2	F: AGGGTGAGACAGGCGAACA R: CCGTTGAGTCCATCTTTGC	184
COL2A1	F: TGGACGCCATGAAGGTTTTCT R: CCATTGATGGTTTCTCCAAACC	142
YWHAZ	F: ACTTTTGGTACATTGTGGCTTCAA R: CCGCCAGGACAAACCAGTAT	94

staining with secondary antibodies. Staining was performed for 1 h in the dark. Then the cultures were triple washed with 0.01 M PBS. The 100 ng/mL DAPI (Sigma Aldrich; USA) was used to ensure staining of the nuclei. Staining was performed for 15 min, then the cultures were triple washed with 0.01 M PBS.

The stained preparations were assessed using the Olympus IX53F fluorescent microscope with four fluorescence filters (Olympus; Japan); morphometry involved the use of the Olumpus cellSens Standard software (Olympus; Japan).

Polymerase chain reaction

To perform PCR analysis of expression, one million of cells were lysed in the RLT buffer (QIAGEN; Germany). RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN; Germany) in accordance with the manufacturer's protocol. The total RNA concentration in the sample was measured using the Infinite 200 Pro microplate reader (Tecan; Switzerland) and the I-control software. The MMLV RT kit (Evrogen; Russia) was used for synthesis of the first cDNA strand from an RNA template. Synthesis was performed according to the manufacturer's protocol. To perform real time PCR, we added 5 μL of 5x qPCRmix-HS SYBR (Evrogen; Russia), 0.8 μL of the 10 μM primer (Table 1), 18.2 μL of water, and 1 μL of template cDNA per well of the 96-well plate (SSIbio, Scientific Specialities; USA). Another 1 μL of water was added to the control wells instead of template cDNA.

The reaction was carried out using the 1000 CFX Manager version C10000 Touch thermal cycler for nucleic acid amplification (Bio-Rad; USA) and the CFX Manager software. The number of cycles was 39. The results were analyzed in Microsoft Excel (Microsoft; USA) by the ΔΔCt method.

Obtaining the primary chondrocyte culture from the donor material

Chondrocytes (hereinafter, Chondro) were isolated from the patient's biopsy (surgical) specimen. The cartilage was washed with 15 mL of DMEM with 2% penicillin/streptomycin (PanEco; Russia). Then the cartilage was placed in the clean Petri dish and chopped with the sterile scissors and scalpel in 4 mL of DMEM with 2% penicillin/streptomycin. It was washed once with the same medium in a 15 mL test tube. The cartilage pieces were incubated for 40 min on a shaker at +37 °C and 5% CO_2 in 10 mL of DMEM with 2% penicillin/streptomycin, collagenase IV (Gibco; USA), and the collagenase enzyme preparation (BioPreparat; Russia) having a concentration of 3000 U/mL.

After incubation the cartilage pieces were centrifuged for 5 min at 200 g, once washed, added 10 mL of the culture medium (DMEM/F12 with 20% FBS, 1% GlutaMax, 1% penicillin/streptomycin), and transferred to the T-75 culture flask pre-treated with the 0.1% gelatin solution. The cartilage pieces were cultured at +37 °C and 5% CO_2 until the chondrocyte monolayer was formed. The medium was changed every three days.

Obtaining the primary fibroblast culture from the donor material

The patient underwent skin biopsy of the forearm. The biopsy specimen was put in the droplet of medium (DMEM (PanEco; Russia) supplemented with 10% FBS and 1% penicillin/streptomycin) on the Petri dish and cut into small pieces (of about 1 mm) with a sharp sterile scalpel. The resulting pieces were places in different 35 mm Petri dishes in 3 mL of the culture medium and pressed by the sterile cover glass (Menzel Glasser; Germany). The medium was changed twice a week. After three weeks the fibroblasts (hereinafter, Fibro) were separated and passaged using the 0.25% EDTA solution (Gibco; USA).

Assessment of PBMC activation and degranulation after their co-cultivation with the chondrocyte-like cells

To obtain the peripheral blood mononuclear cells (PBMCs), 9 mL of blood collected from the healthy donor were 2-fold diluted with PBS, carefully layered onto the Ficoll-Paque™ PLUS with the density of 1.077 g/cm^3 (GE Healthcare; USA), and centrifuged for 30 min at 350 g. The interphase layer was separated and washed twice with PBS. The PBMCs collected were enumerated with the Luna automated counter and diluted with the medium (X-VIVO™ 15 (Lonza; Switzerland) + 100 U/mL IL2 + 10% FBS inactivated by heating) to a concentration of 1 million cells per milliliter.

To perform analysis, each of the target cell lines (iPS, iPS_dB2M, Fibro, Chondro, iCh, and iCh_dB2M) was seeded into three wells of the 96-well plate containing appropriate culture medium. After the monolayer was formed, the medium was removed, and 200 μL of the PBMC suspension were added to the well. The plate was transferred to the incubator at +37 °C and 5% CO_2.

To assess $CD8^+$ T cell activation one day and five days later, the cells were resuspended and collected for further flow cytometry analysis.

To assess $CD8^+$ T cell degranulation, Brefeldin A with a concentration of 100 ng/mL was added to the well after 1 h of incubation, and the cells were incubated at +37 °C and 5% CO_2 for another 4 h. Then the cells were collected for flow cytometry analysis.

Flow cytometry

To assess $CD8^+$ T cell activation, the following antibodies were used: CD3-PERCP-Cy5.5 (Sony; Japan), CD8-BrilliantViolet421 (BD Bioscience; USA), CD69-FITC (Sony; Japan)

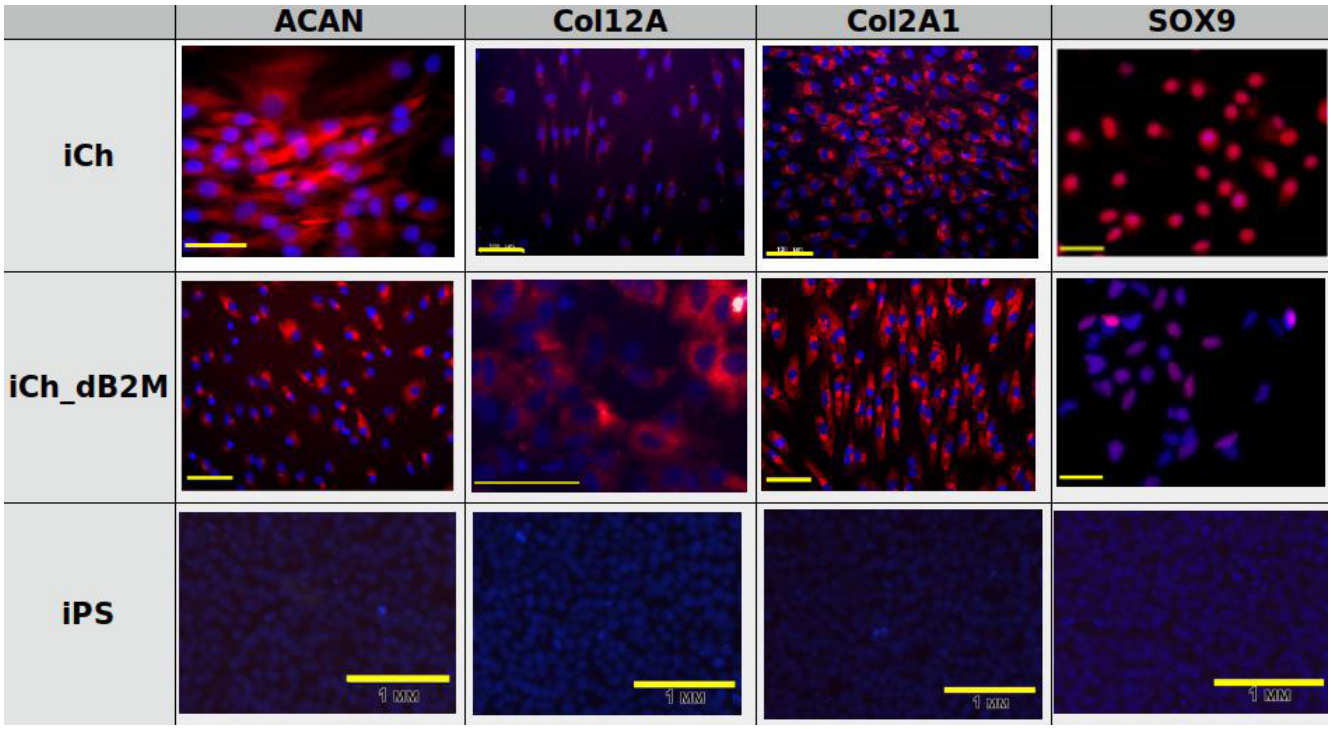

Fig. 1. Immunohistochemical assessment of monolayer cultures. The cell nuclei are stained *blue*, the studied markers are stained *red* and *green*. The scale bar size (shown in *yellow*) is 100 μm for iCh and iCh_dB2M, 1000 μm for iPS

The CD8-BrilliantViolet421 (BD Bioscience, USA), CD3-PE (Abcam, UK), CD107a-APC (Sony; Japan) antibodies were used to assess $CD8^+$ T cell degranulation. Flow cytometry analysis was performed with the NovoCyte Flow Cytometer. The cytometry data were processed using the FlowJo software tool (Tree Star Inc.; USA).

Statistical analysis

The unpaired Student's t-test and ANOVA were used to compare the fractions of activated and degranulated $CD8^+$ T cells. The differences were considered to be significant when *p*-value was below 0.05. Calculation was performed using a personal computer with the Microsoft Excel 2010 (Microsoft Corp; USA) and SPSS Statistics 17.0 (IBM; USA) software.

RESULTS

Quality control of the iPSC-derived chondrocytes

The chondrocyte-like cells obtained by directed differentiation of the B2M knockout and wild-type iPSCs were tested for expression of the major chondrogenic markers. The analysis of immunohistochemical labeling revealed fluorescence of the chondrocyte matrix proteins, type I and II collagens (COL1, COL2), aggrecan (ACAN), and the SOX9 nuclear protein in both cell lines (Fig. 1). However, iPSCs were not stained for these markers.

The real time PCR also demonstrated expression of these chondrogenic markers in both B2M knockout derivatives and wild-type ones. Furthermore, the type I collagen expression in the iCh_dB2M cells was lower than in the control group of native human chondrocytes, while the expression of type II collagen, on the contrary, was higher (Fig. 2). Furthermore, the B2M knockout iPSC derivatives showed lower ACAN and SOX9 expression compared to human chondrocytes, as well as lower fluorescence intensity compared to the cell line without knockout. No significant differences in expression of the studied chondrogenic markers from the control group were reported for the iCh cells.

Assessment of the iPSC-derived chondrocytes' immunogenicity

MHC I (major histocompatibility complex class I) is expressed in almost all cells of the body. Presentation of endogenous peptides to T cells is the main function of this protein. The genes encoding MHC I are highly polymorphic and vary between individuals, just like the set of peptides capable of presenting in appropriate MHC I molecule. During maturation the $CD8^+$ T cells acquire tolerance to their own endogenous peptides and MHC I. However, when foreign cells enter the body, the likelihood of nonspecific recognition of the peptide–MHC I complex by the T-cell receptor is high. That is why attention was paid mainly to the $CD8^+$ T cell response.

To assess the iCh-dB2M immunogenicity, this cell line was co-cultured with PBMCs of a healthy donor. After the 5-day co-cultivation we determined the percentage of activated $CD69^+$-$CD8^+$ T cells. CD69, the membrane-bound type II C-lectin receptor, is widely used as an early marker of lymphocyte activation. The CD69 expression is induced promptly on the T cell surface after binding of the T-cell receptor and CD3, which results in the cytokine secretion and proliferation of activated cells. A number of studies report the fact that CD69 expression on the T cells reaches its maximum 24 h after stimulation and then starts to decline [16, 17]. That is why we conducted another experiment, during which we measured T cell activation 24 h after the start of co-cultivation.

The flow cytometry analysis results are provided in Fig. 3. Fibroblasts of a healthy donor were used as a positive control, while iPSCs were used as a negative control due to their decreased immunogenicity [18]. Regardless of the co-cultivation duration, the percentage of $CD8^+$ T cells activated during co-cultivation with iCh-dB2M did not differ significantly from that observed during co-cultivation with

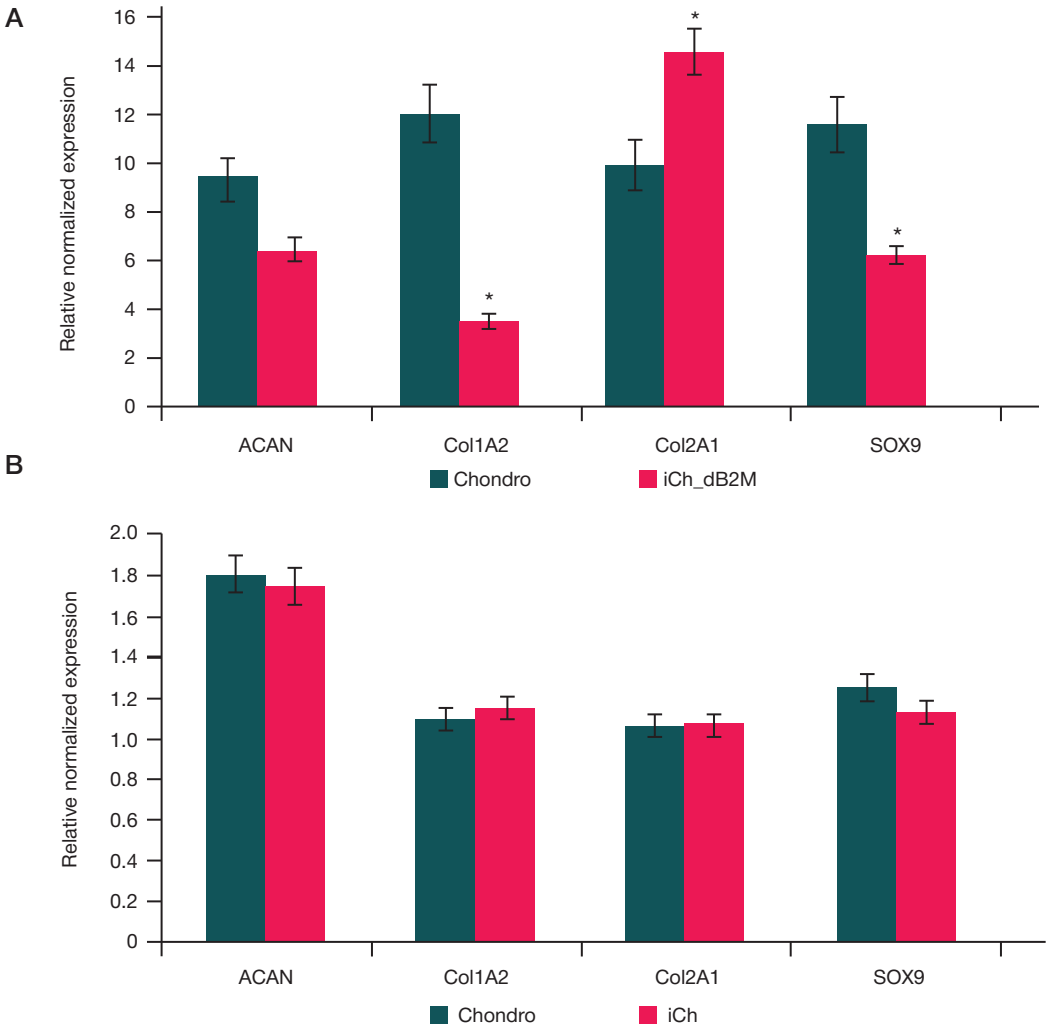

Fig. 2. Relative expression of chondrocytic markers in the human chondrocyte lines (Chondro), B2M knockout (iCh_dB2M) **(A)** and wild-type chondrocyte-like cells (iCh) **(B)**. * — significant differences in gene expression relative to human chondrocytes, $p < 0.05$, unpaired Student's *t*-test

iCh and chondrocytes, but was higher than that observed during co-cultivation with iPS or without co-cultivation. On the one hand, this may indicate that the B2M knockout is not enough for the cells to eventually become hypoimmunogenic [11, 19]. On the other hand, the 5-day co-cultivation of all the cell lines used resulted in activation of the significantly lower percentage of $CD8^+$ T cells ($p < 0.01$, unpaired *t*-test; the data are not provided) compared to fibroblasts, which is probably indicative of decreased immunogenicity of the chondrocyte-like cells and chondrocytes per se. According to the literature data, chondrocytes are capable of creating anti-inflammatory microenvironment around them, which is likely to affect the experimental results [20, 21]. At the same time, the 1-day co-cultivation with fibroblasts of a healthy donor did not result in activation of $CD8^+$ T cells. Perhaps, several factors contributed to such results. First, according to flow cytometry data, the MHC I expression on the fibroblasts of this donor was decreased compared to other fibroblast lines represented in our laboratory (the data are not provided). Second, partial match of the MHC I allele in PBMCs and fibroblasts could affect the experimental results. As a result, in case of 5-day co-cultivation, the cumulative effect resulting in activation of the significantly larger number of $CD8^+$ T cells was observed, while the 24 h stimulation was not enough for this cell line.

Thus, the iPSC-derived chondrocytes are capable of activating $CD8^+$ T cells of PBMCs. Furthermore, the B2M knockout does not have a significant effect on immunogenicity of the iPSC-derived chondrocytes.

To assess immunogenicity of the chondrocyte-like cells, the $CD8^+$ T cell cytotoxic response was also measured during co-cultivation of cell lines with PBMCs.

CD107a (LAMP-1) is a lysosomal-associated membrane glycoprotein. When the $CD8^+$ T cell is degranulated, the lysosomal granule containing the effector lytic molecules fuses with the external membrane of the $CD8^+$ T cell. As a result, the granule content is delivered to the target cell, and CD107a is on the cell surface and becomes available for staining with antibodies, as a cytotoxicity marker.

After the 5 h co-cultivation with the target cell lines and Brefeldin A, PBMCs were removed and stained with antibodies against CD3, CD8, and CD107. After that flow cytometry analysis was performed.

The data obtained are generally similar to the previous two results (Fig. 4). The CD107a expression and, therefore, cytotoxic activity of $CD8^+$ T cells observed in the presence of B2M knockout chondrocyte-like cells, chondrocytes, and fibroblasts was significantly higher ($p < 0.05$; unpaired Student's *t*-test) than that observed in the presence of iPS and no target cell line. This suggests that the cell lines we have selected are immmunogenic. It is interesting that the B2M knockout iPS also caused a cytotoxic response. We have found no similar experimental data in the literature. Given the fact that the similar trend towards an increase in immunogenicity in iPS_dB2M compared to iPS was observed throughout all three experiments, it can be assumed that the results obtained

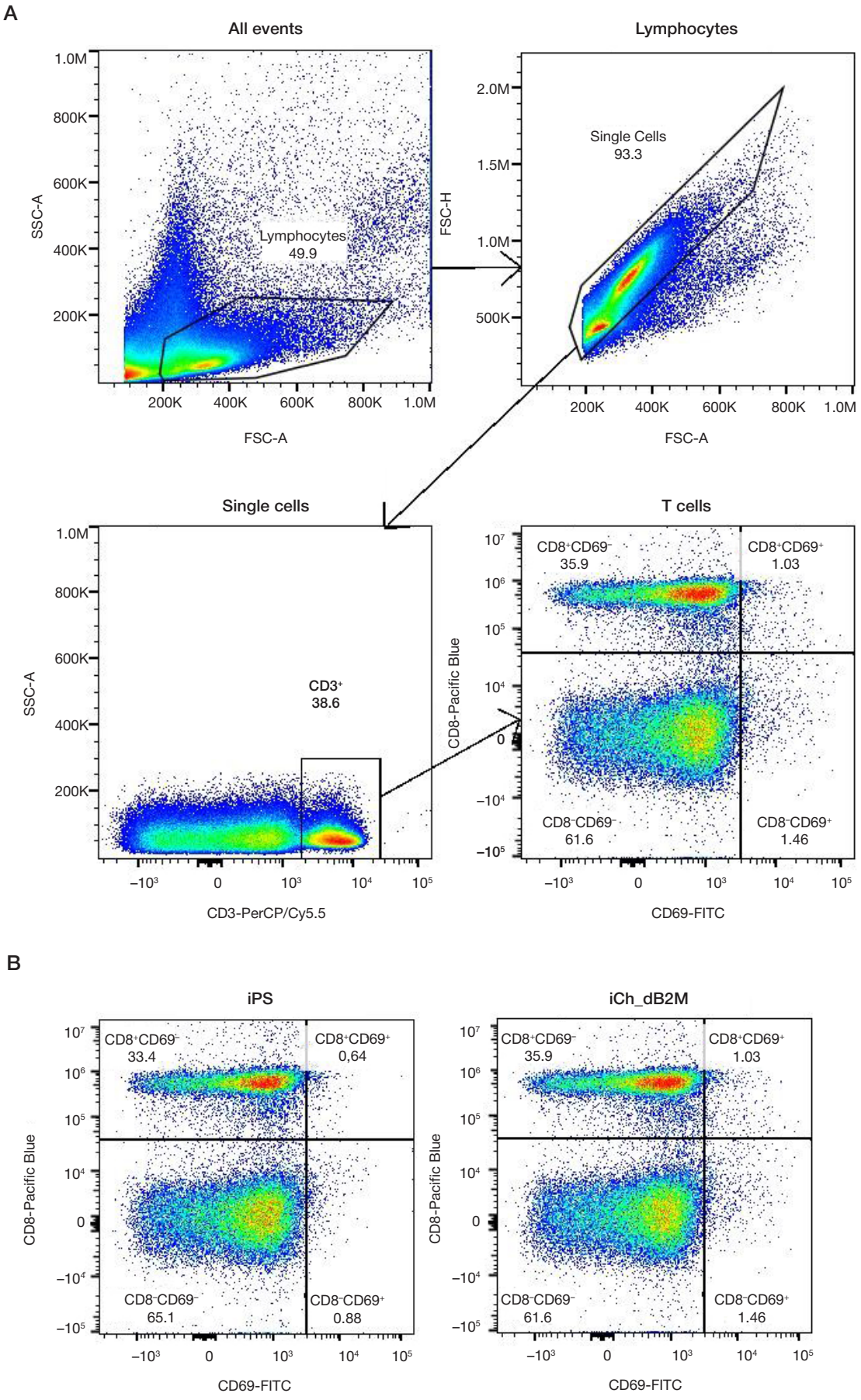

Fig. 3. Flow cytometry analysis of the CD8⁺ T cell activation. **A**. Gating scheme. **B**. Percentage of activated CD8⁺ T cells during co-cultivation with iPS (*on the left*) and iCh-dB2M (*on the right*). **C**. Comparative analysis of CD8⁺ T cell activation after the 5-day co-cultivation with the target cell lines. **D**. Comparative analysis of CD8⁺ T cell activation after the 24 h co-cultivation with the target cell lines. * — $p < 0.05$; ** — $p < 0.01$, unpaired Student's *t*-test

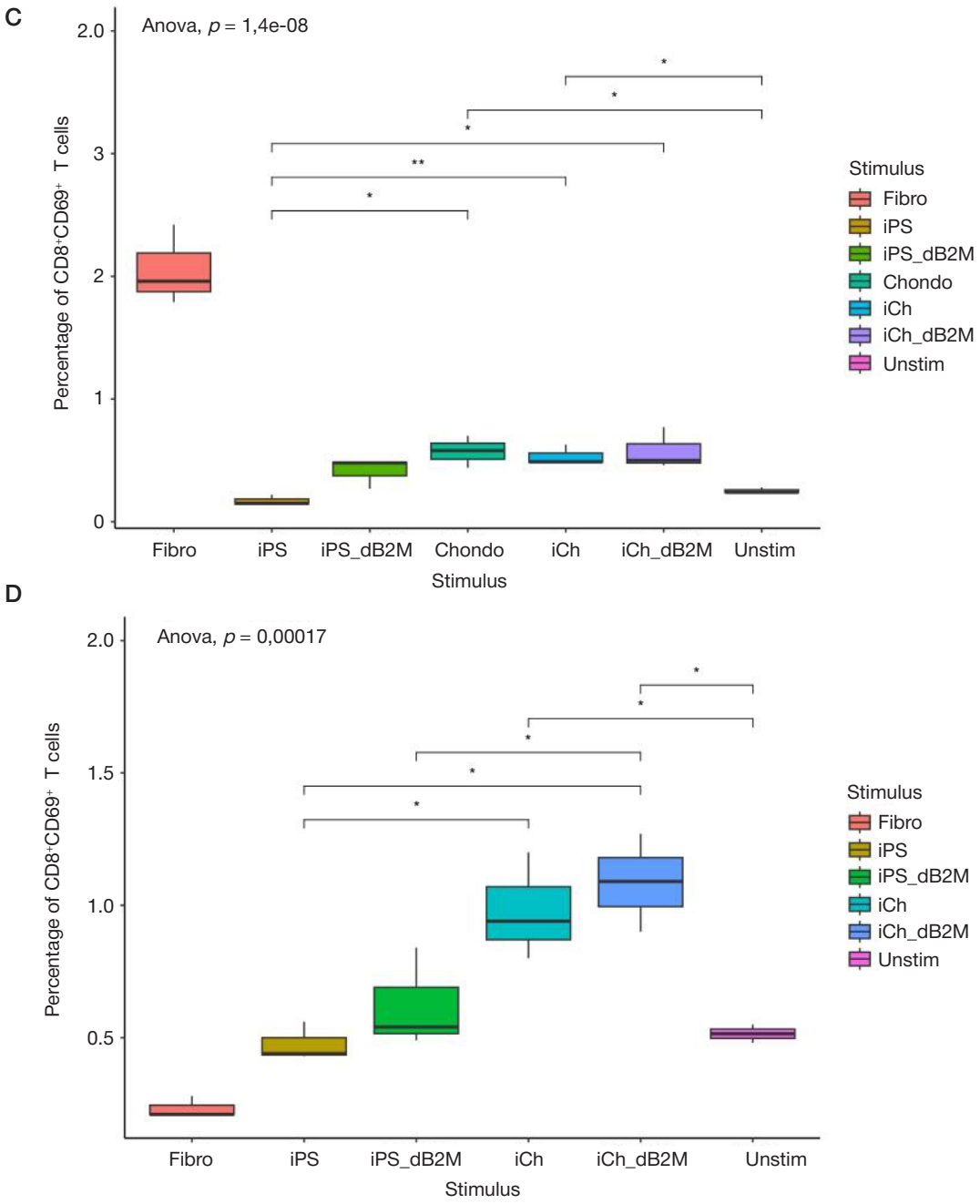

Fig. 3. (Continuation) Flow cytometry analysis of the CD8+ T cell activation. **A**. Gating scheme. **B**. Percentage of activated CD8+ T cells during co-cultivation with iPS (*on the left*) and iCh-dB2M (*on the right*). **C**. Comparative analysis of CD8+ T cell activation after the 5-day co-cultivation with the target cell lines. **D**. Comparative analysis of CD8+ T cell activation after the 24 h co-cultivation with the target cell lines. * — $p < 0.05$; ** — $p < 0.01$, unpaired Student's *t*-test

are not an artifact for this line. Then, the B2M knockout has probably somehow affected the cell line transcriptome [22]. In the future we plan to test the iPS-dB2M transcriptome and compare it to that of the original cell line.

DISCUSSION

The use of cell products for the cartilage tissue repair is currently among the most promising and efficient therapy types [23–25]. However, the limited amounts of the cartilage cell material represent a serious obstacle for the widespread use of such treatment. In recent years, it has become possible to obtain the cartilage cells via differentiation of iPSCs, which has made it significantly easier to obtain the amount of the source of autologous cells essential for creation of the graft. However, this procedure is expensive, it takes several months and requires validation. Creation of the universal hypoimmunogenic graft would help solve the problem of insufficient cell material for *in vitro* creation of the cartilage tissue. Nevertheless, biosafety of such product is poorly understood.

We have obtained the B2M knockout and wild-type iPSC-derived chondrocytes and assessed the expression of chondrogenic markers. The expression of such markers, as SOX9, aggrecan, and type I and II collagens suggests that the iPSC derivatives have acquired a chondrocytic phenotype, which was observed in both lines of derivatives during our experiment. It is worth noting that based on the real time PCR data the expression of Col1 in iCh_dB2M was lower and the expression of Col2 was higher than in human chondrocytes. This was confirmed by predominance of the hyaline cartilage phenotype among the differentiated B2M knockout derivatives, which was more preferable in terms of future clinical use [26]. At the same

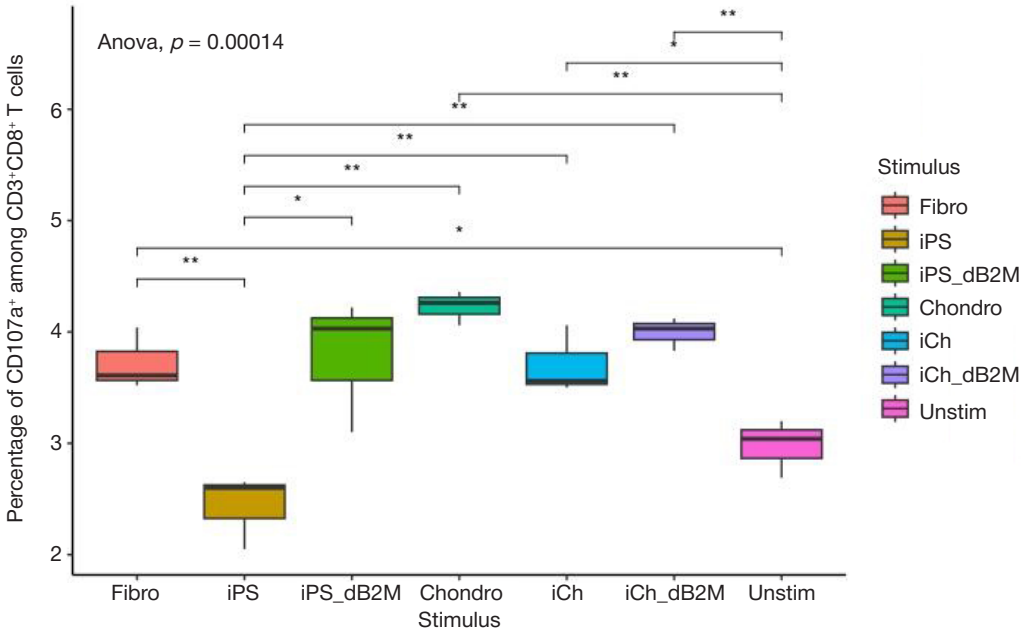

Fig. 4. Comparative analysis of CD8⁺ T cell degranulation after the 5 h co-cultivation with the target cell lines. Unpaired Student's *t*-test, * — $p < 0.05$; ** — $p < 0.01$

time, this could be indicative of acquiring fibrotic characteristics of native human chondrocytes due to cultivation [27]. Despite the fact that the Sox9 expression in iCh_B2M was lower than in iCh, we have no reason to believe that this had a significant effect on the iPSC-derived chondrocytes' functioning [28, 29].

The total removal of MHC I from the cell surface should have resulted in the chondrocyte-like cells' immunogenicity reduced due to escaping recognition of the MHC I–autopeptide complex by the CD8⁺ T cells. However, our findings have shown that knocking out B2M is not enough to reduce immunogenicity of iPSC-derived chondrocytes. In all three of our experiments, the CD8⁺ T cell immune response to the B2M-deficient chondrocyte-like cells was significantly higher than that to iPSCs. There are several mechanisms, which could underlie the CD8⁺ T cell activation.

According to one of those, CD8⁺ T cells were activated not due to direct interaction with the chondrocyte-like cells, but due to the fact that there were antigen-presenting cells (APCs) among PBMCs. As is known, the APCs are capable of presenting peptides of the absorbed particles in the context of MHC I and MHC II. To ensure activation of CD8⁺ T cells (and, therefore, expression of CD69), it is enough to recognize a foreign peptide on the APC and, in some cases, receive co-stimulation from CD4⁺ T cells [30, 31]. As for degranulation, it has been shown that CD8⁺ T cells are capable of killing the tumor cells that have lost MHC I. Such an effect was achieved in the presence of APCs, regardless of NK cells. Recognition is accomplished via binding of the NKG2D T cell receptor with the ligands (NKG2DL) on the target cells, while killing occurs due to granzyme secretion [32]. At the same time, Bogomiakova et al. have shown that the iPS fibroblast derivatives express 1.5 times more NKG2D ligands on their surface than fibroblasts of a healthy human [14]. Thus, it can be assumed that the chondrocyte-like cells used in our experiment increased expression of the NKG2D ligands, which made them a potential target for both CD8⁺ T cells and NK cells. Despite the fact that chondrocytes (and probably iPSC-derived chondrocytes) create the anti-inflammatory environment around them [20, 21], this is probably not an absolute guarantee of no immune response. It must be remembered that chondrocytes that have got into the pro-inflammatory environment (which is inevitable in case of the tissue-engineered cartilage graft transplantation) are capable of expressing MHC II [21]. Despite the fact that it has not been demonstrated *in vitro* for the iPSC-derived chondrocytes [27], no *in vivo* tests have been performed. That is why it is necessary to ensure the MHC II knockout in the iPSC-derived chondrocytes in order to avoid the CD4⁺ T cell immune response. In addition, a number of studies demonstrate successful acquisition of hypoimmunogenic iPS derivatives due to knockout of MHC I, MHC II and CD47 hyperexpression aimed at regulating the NK cell response [11, 12]. These data, in total, leave the room for the possibility of the cartilage tissue graft improvement based on the differentiated iPSC derivatives.

CONCLUSIONS

The iPSC-derived chondrocytes obtained show the same low level of immunogenicity, as human chondrocytes, however, these cause a higher immune response compared to hypoimmunogenic iPSCs. On the one hand, this suggests that the chondrocytes obtained can be a potential source of product for treatment of the articular cartilage tissue, but on the other hand this study demonstrates potential risks associated with instability of tissue after editing and avoidance of the immune response in case of tumorgenesis. Thus, the B2M knockout is not a sufficient condition for immunogenicity of the prototypes obtained from such cells of the tissue, and the iPSC-derived chondrocytes require further modification.

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COMBINED MATRICES AND TISSUE-ENGINEERED CONSTRUCTS MADE OF BIOPOLYMERS IN RECONSTRUCTIVE SURGERY OF ENT ORGANS

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Microtia is a combined congenital malformation with the prevalence of one case per 10,000–15,000 newborns, which accounts for 50% of all congenital malformations. Treatment of microtia is a challenging task. Numerous solutions have been proposed, however, none of these options guarantee good functional and aesthetic outcome. High hopes for solving the problem are placed on advances in reconstructive surgery. The study was aimed to determine the possibility of using advanced biocompatible endoprostheses manufactured using the tissue engineering technologies. Two closely related male 2-year-old minipigs of the Sus salvanius breed underwent implantation of bioengineered implants manufactured by combined 3D bioprinting with application of the collagen solution containing autologous cartilage tissue cells under the temporal fascia. The samples were collected 3 months later. Histological examination and immunohistochemistry showed that the implanted endoprosthesis initiated the development of regenerated connective tissue and its own vasculature in 100% of cases, thereby ensuring cell viability and integrity of biological structures; furthermore, no facts of the endoprosthesis rejection or resorption were reported. We have concluded that the developed implant manufacturing method is promising and can provide the basis for creation of domestic porous ear implants based on biocompatible polymeric materials, hydrogels, and autologous cellular material. It is necessary to further test the auricular implant using biological models.

Keywords: outer ear reconstruction, cell engineering, cartilage tissue, minipigs

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Compliance with ethical standards: animal handling was compliant with the common ethical standards of Basel Declaration.

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КОМБИНИРОВАННЫЕ МАТРИЦЫ И ТКАНЕВЫЕ ИНЖЕНЕРНЫЕ КОНСТРУКЦИИ ИЗ БИОПОЛИМЕРОВ В РЕКОНСТРУКТИВНО-ВОССТАНОВИТЕЛЬНОЙ ХИРУРГИИ ЛОР-ОРГАНОВ

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Микротия — это врожденный комбинированный порок, встречается с частотой один случай на 10 000–15 000 новорожденных и составляет 50% всех врожденных пороков. Лечение микротии — сложная задача, предложено множество вариантов ее решения, однако ни один из них не гарантирует высоких функциональных и эстетических результатов. Большие надежды в решении проблемы возлагаются на достижения регенеративной медицины. Целью исследования было определить возможность применения современных биосовместимых эндопротезов, изготовленных с помощью технологий тканевой инженерии. Двум близкородственным самцам-минипигам двухлетнего возраста породы *Sus salvanius* под височную фасцию были имплантированы биоинженерные импланты, изготовленные методом комбинированной трехмерной биопечати с нанесением на них раствора коллагена с аутогенными клетками хрящевой ткани. Через 3 месяца образцы были изъяты. По результатам их гистологического и иммуногистохимического исследования, в 100% случаев имплантированный эндопротез инициировал развитие соединительнотканных регенератов и формирование собственной сосудистой сети, тем самым обеспечивая жизнеспособность клеток и сохранность биологических структур, при этом отторжения и явлений резорбции эндопротеза не выявлено. Сделан вывод, что разработанный метод изготовления имплантатов перспективен и может послужить основой для создания отечественных пористых ушных имплантатов на основе биосовместимых полимерных материалов, гидрогелей и аутологичного клеточного материала. Необходима дальнейшая апробация импланта ушной раковины на биологических моделях.

Ключевые слова: реконструкция наружного уха, клеточная инженерия, хрящевая ткань, минипиги

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Соблюдение этических стандартов: все манипуляции с животными были проведены в соответствии с едиными этическими нормами Базельской декларации.

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The combined malformation referred to as microtia or aural deformity with the external auditory canal atresia, which includes malformations of the outer and middle ear, has the prevalence of one case per 10,000–15,000 newborns. The patients with this abnormality suffer not only from severe conductive hearing loss, but also from the gross cosmetic defect [1–5].

Treatment of microtia is a challenging task. Numerous solutions have been proposed, from the use of ear prosthesis to the reconstructive surgical procedure involving the use of various surgical techniques and reconstructive materials. Reconstructive head and neck surgery has to address a complex set of challenges including not only restoration of the lost organs, their parts or tissues, and restoration of function, but also aesthetic rehabilitation [6–8].

Three main methods to eliminate microtia are used in surgical practice: the use of autologous rib cartilage, porous polyethylene implant and intraosseous implants for removable prosthetics. The treatment method is selected based on the microtia severity, functional goals of surgical correction, patient's age, wishes of the patient or his/her representatives. Auricular reconstruction involving the use of autologous rib cartilage graft is traditionally carried out on a step-by-step basis, as reported by Tanzer, Brent, Nagata, and Firmin. The alloplastic implants have gained greater acceptance as one more option for ear reconstruction, since alloplastic reconstruction can be performed in younger individuals without any damage to the donor site. Ear reconstruction using porous polyethylene (Su-Por, Omnipore, Medpor, Porex Surgical) is currently considered to be athe standard method for microtia correction in children under the age of 3 years [3, 4, 9].

The National Medical Research Center for Otorhinolaryngology of FMBA of Russia has accumulated rich experience in elimination of microtia since 2014: more than 516 cases involving the use of various methods and materials. The best outcome was obtained when using the auricle made of porous polyethylene heteromaterial as an endoprosthesis. We managed to achieve satisfactory surgical outcome in 80.48% of cases; complications developed in 19.52% of cases, among which in 12.19% of cases partial extrusion of the endoprosthesis helix was reported, and in 7.31% partial resection of the endoprosthesis was required to close the defect due to migration of the defect and its eruption through soft integumentary tissues.

We assessed aesthetic outcomes of auricular reconstruction using the porous polyethylene heteromaterial. In 24.39% of cases, no prominent postauricular fold or sufficiently protruding ear was obtained in the late postoperative period due to scarring of skin grafts and, consequently, pressing the shaped auricle to the skull, which was considered as unsatisfactory outcome. In all other 75.61% of cases, the auricle shaped had clear contours of the helix and antihelix, correctly positioned earlobe, and was arranged symmetrically with respect to the contralateral ear. Thus, we can say that surgical complications occur in 19.52% of cases when using porous polyethylene heteromaterial as the auricular endoprosthesis, while satisfactory aesthetic outcome can be achieved in 75.61% of cases only. The findings suggest the need to search for new methods and materials for elimination of microtia [10, 11].

Reconstructive surgery has been developing rapidly over the years and recently. The area is interdisciplinary, especially in the field of regenerative medicine. Tissue engineering that involves developing the constructs made of specific materials (matrices, scaffolds) and culturing stem cells or tissue-specific cells on these constructs is an important instrument of regenerative medicine [12–19].

The advances of modern regenerative medicine have found use in cardiovascular surgery, traumatology, orthopedics, trachea surgery, abdominal surgery, urology, plastic and aesthetic surgery, otolaryngology, and maxillofacial surgery [7, 18, 20–22].

It is clear that these technologies can be used for treatment of microtia. This motivated us to carry out an experimental study aimed to search for and create new ear endoprostheses using the today's capabilities of domestic regenerative medicine and tissue engineering. The study was aimed to assess the possibility of using the combined matrices and tissue-engineered constructs made of biopolymers for auricular reconstruction.

METHODS

Two closely related minipigs of the *Sus salvanius* breed were used as biological models in the *in vivo* experiment. The average body weight of minipigs was 37.5 kg. Minipigs were kept at the Research Center of Biomedical Technologies of the Federal Medical Biological Agency under stress-free conditions with *ad libitum* access to food and water. The experiment included several phases (Fig. 1).

A modified Cartesian FDM printer was used to manufacture the implant. The technological process of creating the implant consisted of five major steps: 1) creating a 3D model of the auricle; 2) transformation and adjustment of ear topology; 3) preparation of the cellular component; 4) preparation of the collagen hydrogel; 5) manufacturing the implant.

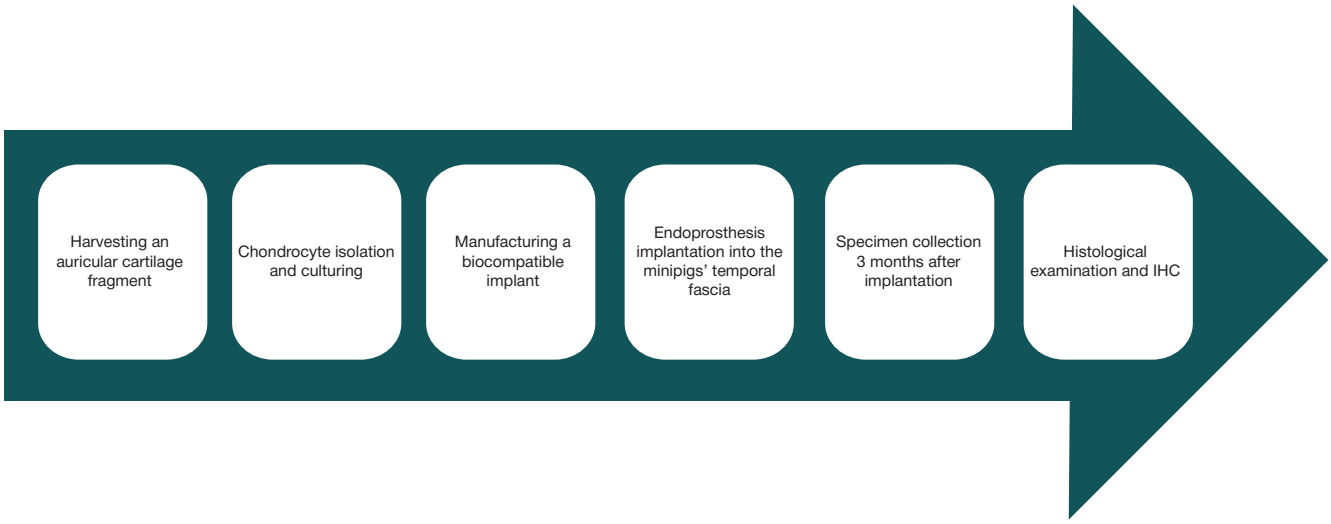

Fig. 1. Flowchart showing phases of the experiment

To obtain autologous chondrocytes from the elastic cartilage, two surgical teams harvested auricular cartilage from the left ears of two animals under total intravenous anesthesia. The cartilage piece size was 1.5 × 1.5 cm. The cartilage pieces were placed in different test tubes with transport medium (buffer). Then chondrocyte isolation from the auricular cartilage was performed followed by cell culturing and cell population expansion; this process took a month. The cells were cultured in the DMEM medium (Gibco; USA) containing 2 mL of L-glutamine (Paneco; Russia) and the single-dose antibiotic antimycotic solution (Gibco; USA) supplemented with 10% (v/v) fetal bovine serum (Gibco; USA) at 37 °C and 5% CO_2. The Versene solution (Paneco; Russia) and the 0.25% trypsin–EDTA solution Gibco; USA) were used to take the cells off the substrate.

After the chondrocyte cultivation, a bioengineered implant printed using two polymers and collagen was manufactured. CT/MRI scans of a healthy patient and the ImageJ (USA) and 3D Slicer (USA) software tools were used to create a 3D model of the auricle. The ear topology transformation and adjustment needed to create a model of the implant were performed using the Autodesk Fusion 360 (USA) and/or KOMPAS-3D (Russia) software tools. The Prusa Slicer and/or Super Slicer (USA) software was used to prepare the model for printing.

The implant was manufactured by combined 3D printing, for which printer was installed in sterile environment. The printer extruders were previously heated to 200 °C, and the stage was heated to 50 °C. Polylactide was used for stiffeners (Ingeo 4032D, China; Natureworks LLC, USA), and the Elastollan 1170 A 10FC000 thermoplastic polyurethane was used for porous scaffold. The polymeric scaffold gyroid pores were filled with the Viscoll collagen gel (Imtek; Russia). We printed layer-by-layer, first with polylactide, then with polyurethane, ensuring that the two polymers were imprinted into each other. After creating the scaffold, a distinct nozzle was used to imprint the hydrogel composition into it. To prepare the collagen hydrogel, dilution and neutralization were performed at a temperature of +4 °C: first, the syringe containing the culture medium (DMEM, 10% fetal bovine serum, 100 mM Tris-HCl) was hermetically connected to the syringe containing the 4% type 1 collagen solution (Viscoll; Russia), in a ratio of 1 : 4. Then the collagen solution was mixed thoroughly with the culture medium to obtain the neutralized homogenous collagen solution.

Furthermore, two pairs of cylinders were prepared (diameter 10.5 mm, length 18 mm). “Cylinder 1” (collagen + autologous chondrocytes) was filled with the specially prepared collagen solution with the swine autologous cartilage tissue cells (chondrocytes, concentration of cells about 30 million per milliliter). “Cylinder 2” (collagen) was not filled; it represented a collagen backbone.

After manufacturing the bioengineered auricles and cylinders 1 and 2 these were implanted under the temporal fascia. Two surgical teams operated both animals simultaneously. The right temporal fascia was detached under total intravenous anesthesia; the auricular implant was installed in the muscle bed and wrapped in the temporal fascia. The left temporal fascia, under which “Cylinder 1” (collagen + autologous chondrocytes) was implanted, was detached in both animals in the same way; “Cylinder 2” (collagen) was implanted under the mobilized frontalis muscle. Thus, three distinct myofascial compartments with implanted bioengineered materials not connected to each other were formed in two animals (Fig. 2).

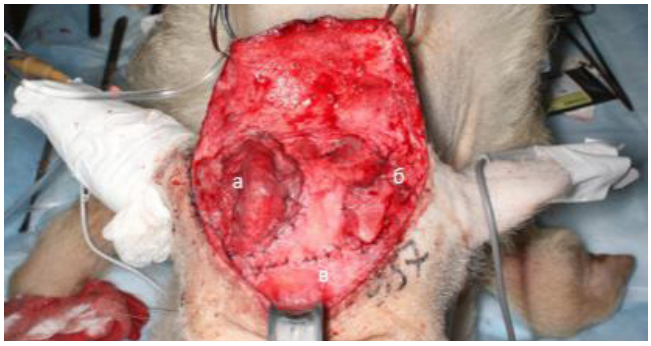

Fig. 2. Surgical wound after implantation of bioengineered ear, “Cylinder 1” and “Cylinder 2”. a — site of the bioengineered ear implantation in the region of the right temporal fascia; b — site of the “Cylinder 1” (collagen + autologous chondrocytes) implantation in the region of the left temporal fascia; c — site of the “Cylinder 12” (collagen) implantation in the region of frontalis muscle

The follow-up period was 3 months. Then both animals were humanely withdrawn from the experiment through intravenous euthanasia. The fragments of implants and disks were collected and fixed in the 10% neutral formalin solution in phosphate buffer. All the implanted materials were sent for histological examination.

RESULTS

After harvesting the implants, initial macroscopic assessment of their condition was performed. Both animals showed complete integration of grafts with their myofascial sheaths, along with no signs of tissue infection or necrosis (Fig. 3).

Almost the same pattern was revealed by further histological examination of bioengineered ears of both animals using the hematoxylin and eosin stain. A moderately dense, thickened area of muscle tissue with abundant fibrovascular connective tissue and blood vessels of varying diameter filled with blood is visible in both specimens. No inflammatory infiltrate has been revealed. The dense, acellular eosinophilic tissue (presumably collagen) adheres closely to the muscle tissue in a multifocal manner. The central part represents a labyrinthine structure of intertwined muscle fibers, among which large fields of eosinophilic fibrous tissue are visible. It should be noted that there is prominent vascularization increasing along the periphery of the central part in both specimens, which is indirect evidence of the graft integration with their myofascial sheaths. Multiple dense lymphoplasmacytic infiltrates showing predominance of plasmacytes are seen in the central part. No neutrophilic inflammation, including the rejection reaction, has been revealed.

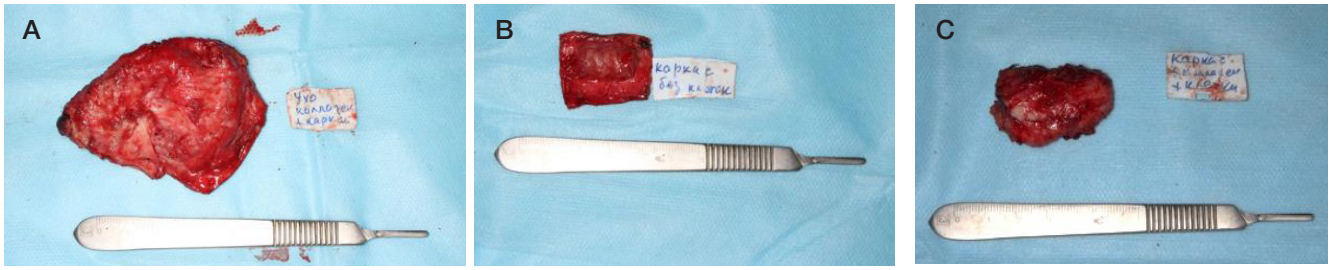

Fig. 3. Bioengineered ear **(A)**, “Cylinder 1” (collagen + autologous chondrocytes) **(B)** and “Cylinder 2” (collagen) **(C)** after harvesting, 3 months after implantation

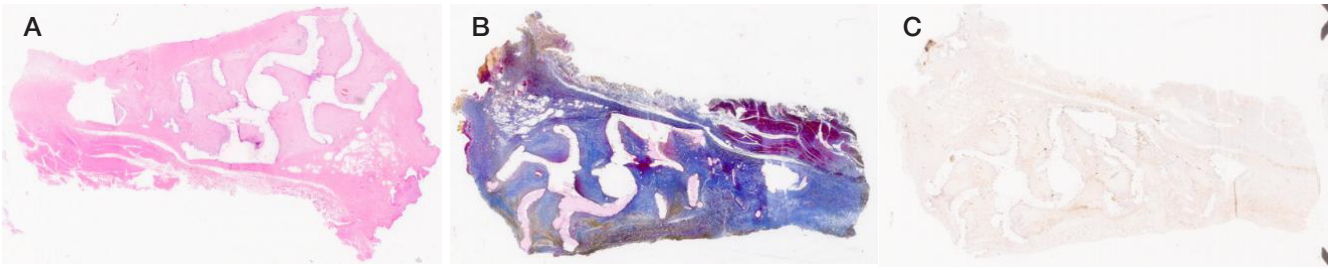

Fig. 4. Histological specimen of bioengineered ear, hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

The similar histological pattern is observed when applying Mallory stain, when large parts of specimens become deep blue, which, according to the staining method, corresponds to collagen fibers. In certain areas, collagen adheres closely to the muscle fibers (brown stain). Immunohistochemistry testing for markers of neoangiogenesis (VEGF and CD31) has revealed weak expression in both specimens (Fig. 4).

Histological examination of the "Cylinder 1" specimen supplemented with type 1 collagen-based hydrogel containing autologous chondrocytes has shown that the specimens consist primarily of loose fibrous tissue with the maze-like voids. In some places there are narrow fields of fibrosis and small roundish fields of acellular eosinophilic tissue. The fibrous tissue is moderately vascularized, with numerous large, slit-like blood vessels filled with blood. Multifocal inflammatory lymphoplasmacytic infiltrates have been revealed, along with the development of lymphoid follicles consisting mainly of small-sized lymphocytes. It is noteworthy that a narrow layer of mature cartilage tissue can be seen between the muscles. When applying Mallory stain, no more than 75% of both specimens become deep blue. The color intensity suggests that the collagen content is higher on the periphery. In the center light blue, red, and blue colors can be seen, which suggests the presence of collagen fibers between the muscle bundles, on the periphery of specimen, outside the implant area. Immunohistochemistry testing for expression of VEGF and CD31 markers has revealed strong expression in the vascular endothelium (Fig. 5).

Histological examination of the "Cylinder 1" stained with hematoxylin and eosin has revealed predominance of the tissue fragments consisting entirely of the moderately dense, fibrous tissue and containing the maze-like voids. The fibrous tissue and scanty vascularization are visible in the middle part of these tissues, along the periphery. Dense lymphoplasmacytic sheaths can be seen around the blood vessels. In the central part, there are multiple dense mixed-cell infiltrates showing predominance of plasmacytes and the presence of sporadic lymphocytes, even smaller neutrophil counts and varying siderophage counts. When applying Mallory stain, large parts of both specimens acquire blue color of varying intensity, depending on the field of view, which suggests high predominance of collagen fibers. Immunohistochemistry has revealed weak expression of the neoangiogenesis markers (VEGF and CD31) (Fig. 6).

Comparative characteristics of the histological specimens of bioengineered ear, "Cylinder 1" and "Cylinder 2" obtained from both animals are provided in Table 1.

The analysis of data provided in Table 1 suggests that the construct manufactured contributed to the development of the regenerated connective tissue having its own vasculature, while the structure of the regenerated tissue fiber backbone was adjusted to the construct shape, which ensured the joint response to the exposure to external mechanical forces. There were no signs of scaffold rejection. Filling the scaffold with cells and collagen affected the regenerated tissue structure; the regenerated tissue filled the entire implant in all cases.

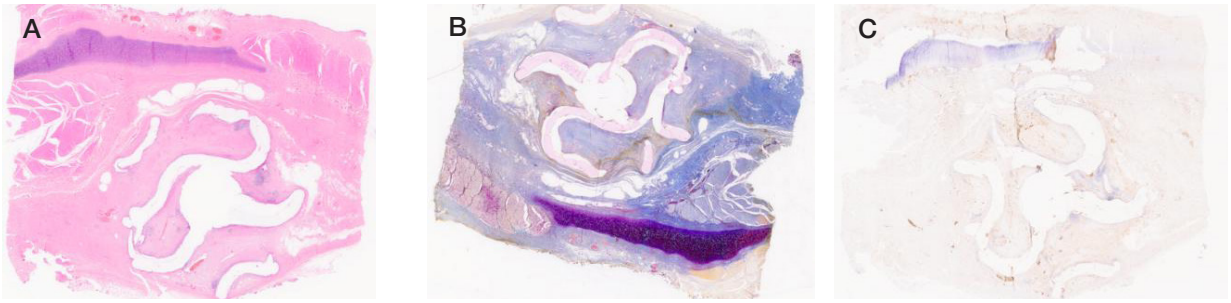

Fig. 5. Histological specimen of "Cylinder 1" based on type 1 collagen with autologous chondrocytes: hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

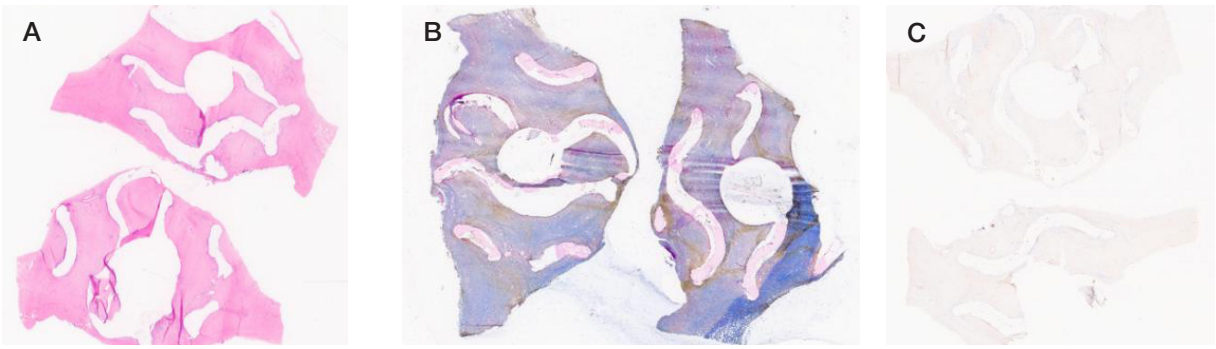

Fig. 6. Histological specimen of "Cylinder 2": hematoxylin and eosin stain **(A)**; Mallory stain **(B)**; immunohistochemistry specimen **(C)**

Table. Comparative characteristics of histological specimens obtained from both animals

	Animal 1			Animal 2		
	Auricle	Cylinder 1 (collagen + chondrocytes)	Cylinder 2 (polylactide)	Auricle	Cylinder 1 (collagen + chondrocytes)	Cylinder 2 (polylactide)
Vascularization	++–	++–	+– –	++–	++–	+– –
Integration and adhesion	+++	+– –	+– –	+++	+– –	+– –
Angiogenesis markers VEGF and CD31	+– –	+++	+– –	+– –	+++	+– –
Signs of inflammation and rejection	– – –	+– –	+– –	---	+– – –	+– –

Note: +++– — parameter is strongly expressed; ++– – — parameter is moderately expressed; +– – — parameter is weakly expressed; – – – — parameter is not expressed.

DISCUSSION

The volumetric product representing a combined tissue-engineered scaffold in the shape of the auricle was designed based on the digital 3D model of human auricle. The scaffold was effectively reproduced by polymer 3D printing and used in the experiment involving minipigs. The product unique nature and dimensionality result from the features of the arrangement of polylactide stiffeners following the contours of the auricular tragus, antitragus and helix, as well as from the shape and arrangement of polyurethane gyroid meshes. Imprinting of mesh threads into polylactide has made it possible to avoid sagging of the meshes and obtain the mechanically strong polymeric scaffold suitable for implantation into a living organism. The interconnected open spaces are located between the threads of gyroid meshes. These open spaces can be artificially filled with the collagen-based hydrogel, hydrogel with living cells; the open spaces turned out to be accessible for natural spontaneous ingrowth of regenerated surrounding tissues after the scaffold implantation under the temporal fascia of the living body.

The use of such technologies has made it possible to solve a series of technical problems reported in many foreign studies, which are related to the contour path control, mechanical strength, and stability of the bioengineered endoprosthesis shape [21].

Scaffold implantation under the temporal fascia of the model animal initiated the development of the regenerated connective tissue growing from the connective tissue anatomical structures (primarily temporal fascia) surrounding the implant. The regenerated tissue inside the implant had its own vasculature ensuring viability of cells and preservation of biological structures throughout the entire volume of the auricular scaffold.

The scaffold structure determined the possibility of growth and the features of the regenerative and adaptive remodeling of live tissues and blood vessels filling the scaffold. Successful integration of the non-absorbable scaffold obtained into the living organism results largely from mechanical characteristics of the implant (the scaffold functions as the inner “skeleton” of the ear) matching the characteristics of the live tissues it is attached to. The findings suggest the effectiveness of combining stiffeners with gyroid meshes. It has been found that the structure of the regenerated tissue fibrous backbone adapts to the construct shape, thereby ensuring the joint response of the tissue-engineered construct that makes it possible to preserve integrity of the live tissues and scaffold when exposed to external mechanical forces.

It has been found that filling the scaffold with cells and collagen affects the regenerated tissue structure. This can be taken into account when further improving the tissue-engineered construct of the auricle and developing new methods for implantation of the construct and auricular reconstruction. The regenerated tissue filled the entire interpolymeric volume of the scaffold in all cases, and ensuring filling the construct with the elastic cartilage may constitute the goal of further research.

CONCLUSIONS

The *in vivo* studies involving biological models have shown that the scaffold shaped like a full-size auricle is superior to the control samples due to ensuring better integration and interplay with live tissues, no inflammation, and vascularization that is sufficient for survival of tissues located within the tissue-engineered construct. It is necessary to further test the auricular implant using biological models. The implant manufacturing method developed is promising and can form the basis for developing domestic porous ear implants based on biocompatible polymeric materials, hydrogels, and autologous cellular material.

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ASSOCIATION OF *GSTP1* GENE WITH RENAL FUNCTION IN PATIENTS WITH DIABETES MELLITUS

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Introduction of point genetic associations into clinical and laboratory diagnosis will allow the physician to determine the risk of severe diabetes mellitus and its complications with a focus on detection of the genetically determined disorder. The study was aimed to identify the molecular genetic markers of severe diabetic nephropathy in patients with type 1 and 2 diabetes mellitus (DM) based on the *GSTP1* (*I105V*) gene assessment. Genotyping of the *GSTP1* gene *I105V* locus was performed in patients with type 1 and 2 DM. Then we identified the features of oxidative status, free radical oxidation, and renal function in patients with various polymorphic variants of the studied gene. Patients with type 1 DM, who were carriers of the *GSTP1* heterozygous polymorphic variant (*Ile/Val*), showed higher activity of the oxidative stress enzymes (glutathione-S-transferase, catalase) and malondialdehyde compared to homozygous carriers ($p < 0.001$, $p < 0.001$, $p < 0.05$). They also showed a significant increase in the levels of triglycerides (1.6-fold) and the glycated hemoglobin levels (1.1-fold) ($p < 0.05$). Patients with type 2 DM, who were carriers of the *GSTP1* polymorphism homozygous for allele 2 (*Val\Val*), had a higher level of malondialdehyde (100.5 μmol/L, ($p < 0.001$)), which was associated with the more severe diabetic nephropathy (average glomerular filtration rate — 48 mL/min/1.73 m2, 24-h urinary albumin excretion — 0.9 g/L; $p < 0.01$). It has been proposed to assess the *GSTP1* (*I105V*) gene in individuals with type 1 and 2 DM. This polymorphism that is heterozygous in individuals with type 1 DM and homozygous for allele 2 in individuals with type 2 DM is unfavorable in terms of the DM course and complications.

Keywords: diabetic nephropathy, oxidative stress, *GSTP1* (*I105V*) gene, personalized medicine

Author contribution: Kostyushok NYa — preparation of tests, experimental procedure, analysis of the results; Gornov SV — research management, manuscript editing; Sizov AV — manuscript revision.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Kuban State Medical University (protocol № 91 dated 29 September 2020). All patients submitted the informed consent to study participation.

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СВЯЗЬ ГЕНА *GSTP1* С ФУНКЦИОНАЛЬНЫМ СОСТОЯНИЕМ ПОЧЕК У БОЛЬНЫХ САХАРНЫМ ДИАБЕТОМ

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Введение в клинико-лабораторную диагностику точечных генетических ассоциаций позволит врачу определять риск тяжелого течения диабета и его осложнений, делая упор на выявление генетически детерминированного патологического состояния. Целью работы было выявить молекулярно-генетические маркеры тяжелого течения диабетической нефропатии у пациентов с сахарным диабетом (СД) 1-го и 2-го типа на основании изучения гена *GSTP1* (*I105V*). Проводили генотипирование локуса *I105V* гена *GSTP1* у пациентов с СД 1-го и 2-го типа. Далее выявляли особенности окислительного статуса, свободнорадикального окисления и функции почек у пациентов с различными полиморфными вариантами исследуемого гена. Пациенты с СД 1-го типа — носители гетерозиготного варианта полиморфизма (*Ile\Val*) гена *GSTP1* — имели более высокий уровень активности ферментов окислительного стресса (глутатион-S-трансферазы, каталазы) и малонового диальдегида по сравнению с гомозиготными носителями ($p < 0{,}001$, $p < 0{,}001$, $p < 0{,}05$). У них также выявлено значимое повышение уровня триглицеридов в 1,6 раз и повышение уровня гликированного гемоглобина в 1,1 раз ($p < 0{,}05$). Пациенты с СД 2-го типа — носители гомозиготного по аллелю 2 полиморфизма (*Val/Val*) гена *GSTP1* — имели более высокий уровень малонового диальдегида (100,5 мкмоль/л, ($p < 0{,}001$)), что сочеталось с более тяжелым течением диабетической нефропатии (среднее значение скорости клубочковой фильтрации — 48 мл/мин/1,73 м^2, уровень суточной альбуминурии — 0,9 г/л; $p < 0{,}01$). Предложено производить анализ гена *GSTP1* (*I105V*) у лиц с СД 1-го и 2-го типа. Данный полиморфизм в гетерозиготном состоянии у лиц с СД 1-го типа и в гомозиготном по аллелю 2 состоянии у лиц с СД 2-го типа неблагоприятен в отношении течения СД и его осложнений.

Ключевые слова: диабетическая нефропатия, окислительный стресс, ген *GSTP1* (*I105V*), персонифицированная медицина

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The world's clinical diagnostic laboratories (CDLs) are gradually moving toward a personalized medicine [1], which represents a modern approach to health protection considering specific features of each patient, including genetic markers and variable phenotypic traits. Such an approach makes it possible to more accurately diagnose the disease, to select optimal treatment and prevention methods, to reduce the risk of complications [2]. Introduction of the personalized medicine and specialized

CDLs will enable improving the quality of medical care, reducing the costs of public health services, and improving the patients' prognosis [3].

When discussing personalized medicine, we cannot ignore a disorder that is called the non-infectious pandemic of the 20th and 21st centuries. In 2022, there were almost 9 million people suffering from diabetes mellitus (DM) all over the world [4]. Furthermore, the increase in the number of individuals suffering from both type 2 and type 1 DM is reported [5]. It is possible to prevent type 2 DM or alleviate its course and complications through adjustment of lifestyle and diet, increase in physical activity. However, we still cannot affect the development of type 1 DM. Cardiovascular disorders, the risk of which increases 2–4-fold after the onset of diabetic nephropathy (DN), represent the main cause of mortality among patients with type 1 and type 2 DM [6, 7]. DN is a terrible complication of DM, because clinical symptoms manifest themselves at the latest stages only. According to the research, the early-stage chronic kidney disease (CKD) is missed in 20% of patients [8]. DN in patients with type 1 DM manifests itself within 10 years after the disease onset on average. In case of DM decompensation, this median is shifted, and the CKD progression takes place earlier [9]. In patients with type 2 DM, it is recommended to estimate DN severity immediately when making the diagnosis of DM. This is associated with the fact that due to mild manifestation of type 2 diabetes, patients can have hyperglycemia for a long time, which adversely affects the renal function. Inability to use modern nephroprotective drugs (sodium-glucose cotransporter 2 (SGLT-2) inhibitors; glucagon-like peptide-1 agonists, thiazolidinediones) slowing down the CKD progression is an important problem of patients with type 1 DM. The use of such drugs in patients with type 1 DM has not been sufficiently studied and is currently contraindicated [10]. As for individuals with type 2 DM, the use of nephroprotective drugs, on the contrary, has a beneficial effect on the course of DN, since these drugs maintain the glomerular filtration rate (GFR) and reduce albuminuria [11]. The methods to estimate severity of the diabetic complications, specifically DN, are used in the clinical laboratory diagnosis. However, all these methods (determination of the renal function of filtration through calculation of creatinine levels, albumin-to-creatinine ratio (ACR) in a single urinary specimen, micro- or macroalbuminuria in 24 h urine, cystatin C, etc.) become effective after the CKD onset. There are no clear markers warning physicians about the likelihood of severe diabetes and its complications, specifically DN. In our opinion, the molecular genetic markers can inform the physician about the diabetes severity. It is important to choose the genes capable of affecting the course of DN with high sensitivity and specificity amongst the multitude of genes. The leading diabetologists of our country have described the importance of the search for polygenic associations instead of individual genes and have identified the genes responsible for antioxidant protection as the candidate genes [12]. Introduction of point genetic associations into clinical laboratory diagnosis will allow the physician to determine the risk of severe diabetes and its complications, prevent these complications in a timely manner, intensify the glucose-lowering therapy, increase the number of the patients' preventive check-ups focusing on prevention and identification of the specific genetically determined complication. This represents a fundamental link of the personalized medicine in general and the personalized clinical laboratory diagnosis in particular.

When performing the search for probable candidate genes involved in the DN pathogenesis, we became interested in the role of the *GSTP1* (*I105V*) gene. This gene encodes the glutathione-S-transferase (GST) enzyme being one of the main contributors to the xenobiotic transformation process. Normally, GST contributes to the glutamate interaction with the electrophilic nitrogen (N), carbon (C), sulfur (S) and oxygen (O) atoms and ensures conjugation of sulfhydryl groups with the xenobiotic molecules. The detoxification process driven by GST is the key process of protecting cells from lipid peroxidation and protein alkylation, which increases the resistance to hypoxic states [13]. The *GSTP1* gene *I105V* (A>G) polymorphism is associated with the adenine (A) nucleotide substitution with guanine (G), which results in substitution of the amino acid in the enzyme peptide chain, thereby reducing the enzyme activity and, consequently, increasing accumulation of free radicals in the body. The increased risk of various forms of lung cancer and oral cancer is observed in the G/G genotype carriers [14]. This polymorphism is also associated with susceptibility to leukemia and Parkinson's disease. There are deletion polymorphisms (*GSTM* (*del*), *GSTT1*) that determine the nonfunctional null alleles. It is assumed that individuals with these deletions in homozygous state have a decreased ability to detoxify chemical substances. Such polymorphisms are most often found in women with endometriosis and individuals with allergy [15]. Recently, it has been found out that there are studies, in which the *GSTP1* (*I105V*) gene affects the drug pharmacokinetics, along with the genes of the P-glycoprotein transporter (MDR1), organic cations (OCT1), and organic anions (OATP-C, OAT1, OAT3) [16].

The study was aimed to identify the molecular genetic markers determining the severity of diabetic nephropathy in patients with type 1 and 2 DM based on the *GSTP1* (I105V) gene assessment.

METHODS

The study was conducted at the Department of Endocrinology of the Faculty of Advanced Training and Retraining of Specialists of the Kuban State Medical University. Patients were enrolled at the Regional Clinical Hospital of Emergency Medical Care (Krasnodar). A total of 51 individuals with type 2 DM and 49 individuals with type 1 DM were included in this open-label prospective cohort study. Inclusion criteria: patients' age 20–60 years; type 1 and 2 DM duration 10–15 years; glycated hemoglobin levels of 7.0%–9.5%; glomerular filtration rate exceeding 45 mL/min/1.73 m^2, regardless of the 24 h urinary albumin excretion rate; using drugs having no nephroprotective effects (biguanides; sulfonylureas; dipeptidyl peptidase-4 inhibitors; insulin) as glucose-lowering therapy; no severe comorbidities at the time of the study. Individuals, who did not meet these criteria, were excluded from the study. We also formed the control group of 20 conditionally healthy donors (13 females and 7 males), who were not related to the index group patients and had no history of DM or kidney disease.

Clinical laboratory testing involved the serum samples obtained by centrifuging the whole blood-containing tubes at 3000 rpm and room temperature. The fasting blood glucose levels, postprandial glycemia, complete blood counts, and blood biochemistry were assessed using the Konelab analyzer (Thermo Fisher Scientific; Finland); urinalysis and 24-h urine tests were performed using the SYNCHRON CX9 PRO biochemical analyzer (Beckman Coulter; USA) by immunoturbidimetry. GFR (mL/min/1.73 m^2) was estimated by calculation using the CKD-EPI formula.

The balance of the pro-/antioxidant system of surveyed patients and the control group was assessed based on the activity of the antioxidant defense system enzymes (superoxide

Table 1. Comparison of the *GSTP1* (*I105V*) allele frequencies in the studied groups

Genetic variant	Type 1 DM	Type 2 DM	χ^2	*p*	OR (95%CI)
GSTP1 (*Ile105Val*)					
Homozygote 1 (*ILE/ILE*), %	65.3 (32/49)	62.7 (32/51)	6.572	0.039	0.895 (0.395–2.026)
Homozygote 2 (*VAL/VAL*), %	0 (0/49)	11.8 (6/51)			–
Heterozygote (*ILE/VAL*), %	34.7 (17/49)	25.5 (13/51)			0.644 (0.272–1.524)

dismutase (SOD) [17], catalase (CAT) [18], glutathione-S-transferase (GST) [19] and malondialdehyde (MDA) levels [20]). SOD activity was determined based on the ability to inhibit autoxidation of epinephrine in the alkaline environment. The reaction rate was measured by spectrophotometry based on the resulting absorbance of the released epinephrine autoxidation products in the test sample and relative to the data obtained when there was no epinephrine in the studied blood sample. CAT measurement in the hemolysate was performed by photometry based on the ability to disrupt H_2O_2. The essence of the GST determination method was the ability of reduced glutathione (present in the substrate — 1% hemolysate of patient's red blood cells) to bind 1-chloro-2,4-dinitrobenzene, forming a stable chromogenic conjugate in the alkaline environment. The qualitative assay used to estimate MDA levels in the hemolysate involved adding thiobarbituric acid in the presence of chloroacetic acid.

The *GSTP1* (*I105V*) molecular genetic testing was performed in the molecular genetic research laboratory of the Kuban State Medical University (Krasnodar). The real-time polymerase chain reaction (qPCR) and the RotorGene real-time PCR cycler (QIAGEN; Germany) were used to perform genotyping of the *GSTP1* gene *I105V* locus from the leukocyte fraction. Two amplification reactions with the extracted DNA sample were simultaneously launched (with two pairs of allele-specific primers). The cycler automatically detected the amplification products in each amplification cycle. According to the data obtained, the control program plotted the fluorescent signal accumulation curves for the channel specified for the sample. The analysis results allowed us to draw conclusions of three types: homozygote for allele 1; heterozygote; homozygote for allele 2.

Statistical analysis

Significance of differences in the genotype frequency distribution between the groups of patients with DM and healthy individuals was assessed using the χ^2 test, and the quantitative indicators of the patients' clinical characteristics were assessed using the Student's t-test. Calculations were performed using the BIOSTAT software. The differences were considered significant at $p < 0.05$. To determine compliance with the Hardy–Weinberg principle, we calculated frequencies of all allele variants and the corresponding χ^2 values. The critical χ^2 value exceeded the estimates calculated for each group, suggesting that the Hardy–Weinberg equilibrium was preserved.

RESULTS

The following percentages of the *GSTP1* (*I105V*) polymorphism carriers were revealed among patients with type 2 DM: 25.5% were heterozygous carriers (*ILE/VAL*), 62.7% were carriers homozygous for allele 1 (*ILE/ILE*), and 11.8% were carriers homozygous for allele 2 (*VAL/VAL*). Among patients with type 1 DM, the share of patients with heterozygous gene variant (*ILE/VAL*) was 34.7%; 65.3% were homozygous for allele 1 (*ILE/ILE*); no patients homozygous for allele 2 were revealed. These data are significantly different from that of the control group, where 65% were heterozygous carriers (*ILE/VAL*), 35% were homozygous carriers (*ILE/ILE*), and where there were no carriers homozygous for allele 2 (*VAL/VAL*) (Table 1).

Then we launched the study of the relationship between the renal function and the studied gene polymorphism variant. No differences in GFR and 24 h urinary albumin excretion rate between the heterozygous carriers (ILE/VAL) and the carriers homozygous for the GSTP1 allele 1 (*ILE/ILE*) were revealed among patients with type 2 DM. The average GFR in the subgroups of individuals with type 2 DM having homozygous and heterozygous polymorphisms was 64 mL/min/1.73 m^2, and the average protein level in the 24 h urine was 0.18 g/L. However, patients with type 2 DM, who were carriers of the rare homozygote for allele 2 (*VAL/VAL*), had much worse indicators: the average GFR of this subgroup was 48 mL/min/1.73 m^2, and the 24 h urinary albumin excretion rate was 0.9 g/L. There were no significant differences in other blood biochemistry indicators between the studied gene allele variants in individuals with type 2 DM.

Assessment of the same indicators in the homo- and heterozygous carriers of the *GSTP1* (*Ile105Val*) polymorphisms in the group of patients with type 1 DM revealed no significant differences in the renal function indicators (GFR and 24 h urinary albumin excretion rate). However, a significant (1.6-fold) increase in the levels of triglycerides and a 1.1-fold increase in the glycated hemoglobin levels in the heterozygous carriers (*ILE/VAL*) of the gene compared to homozygous carriers (*ILE/ILE*) were revealed ($p < 0.05$) (Table 2).

No comparison of renal function between patients with type 1 DM, patients with type 2 DM, and the controls was performed, since the control group consisted of healthy individuals.

In the next phase we proceeded to assessing the activity of the antioxidant defense (AOD) enzymes and MDA levels as a function of the studied gene polymorphism. The most significant increase in the activity of the AOD enzymes (catalase

Table 2. Features of changes in the lipid profile indicators and glycated hemoglobin levels in patients with type 1 DM having various *GSTP1* (*I105V*) allele variants

Gene	Group	Glycated hemoglobin	VLDL	Triglycerides	Cholesterol
GSTP1 (*I105V*)	Heterozygote (*ILE/VAL*)	9.41* ± 2.13	4.07 ± 1.46	2.85 ± 1.24**	5.14 ± 0.88
	Homozygote 1 (*ILE/ILE*)	8.74 ± 2.30	3.36 ± 1.15	1.75 ± 0.67	5.00 ± 1.33

Note: * — differences between the group of patients with type 1 DM having the gene polymorphism in the heterozygous state and the patients with type 1 DM having the gene polymorphism in the homozygous state (homozygote for allele 1) at $p < 0.05$ (** — $p < 0.01$, *** — $p < 0.001$).

Table 3. Indicators of the antioxidant defense system and lipid peroxidation in individuals with various *GSTP1* (*Ile105Val*) polymorphic variants

Ile105Val	MDA (μmol/L)	CAT (nmol H_2O_2/mg Hb)	GST (μmol /min/mg of protein)	SOD (AU)
Patients with type 2 DM, genotype (*Ile/Val*), *n* = 14	24.6 ± 2.78* *p* < 0.001	40.3 ± 4.17* *p* < 0.05	30.0 ± 3.2* *p* < 0.05	81.8 ± 4.6* *p* < 0.05
Patients with type 2 DM, genotype (*Ile/Ile*), *n* = 31	34.90 ± 2.5** *p* < 0.001	40.7 ± 1.6** *p* < 0.001	42.2 ± 2.3** *p* < 0.001	89.1 ± 2.2** *p* < 0.001
Patients with type 2 DM, genotype (*Val/Val*), *n* = 5	100.5 ± 4.7*** *p* < 0.05	42.1 ± 8.3*** *p* < 0.001	29.6 ± 6.9*** *p* < 0.05	89.2 ± 8.4*** *p* < 0.05
Patients with type 1 DM, genotype (*Ile/Val*), *n* = 17	37.23 (32.37; 40.90) ### *p* < 0.001	43.36 (37.03; 51.97) # *p* < 0.05	53.02 (44.26; 59.08) ### *p* < 0.001	88.44 (81.10; 98.74)
Patients with type 1 DM, genotype (*Ile/Ile*), *n* = 32	23.30 (17.36; 28.05)	38.04 (27.82; 43.28)	29.41 (23.69; 40.38)	87.20 (73.09; 99.75)
Control group, genotype (*Ile/Val*), *n* = 13	6.4 ± 0.5	30.5 ± 1.6	28.1 ± 1.33	75.0 ± 3.07
Control group, genotype (*Ile/Ile*), *n* = 7	5.8 ± 0.49	33.08 ± 3.4	32.5 ± 3.15	73.8 ± 3.08

Note: MDA — malondialdehyde; SOD — superoxide dismutase; CAT — catalase; GST — glutathione-S-transferase. * — compared to heterozygous carriers of the studied gene in the control group; ** — compared to homozygous carriers of the studied gene in the control group; *** — compared to the indicators of the index group of genotypes (*Ile/Val*) and (*Ile/Ile*). # — differences between the group of patients with type 1 DM having the gene variant in the heterozygous state (*Ile/Val*) and the patients with type 1 DM having the gene variant in the homozygous state (*Ile/Ile*) at $p < 0.05$ (## — $p < 0.01$; ### — $p < 0.001$).

and superoxide dismutase) among patients with type 2 DM was reported for the carriers homozygous for allele 1 compared to heterozygous carriers of the *GSTP1* polymorphisms and the carriers homozygous for allele 2. However, the levels of the advanced glycation end products (MDA) in the subgroup of carriers of the rare homozygote for allele 2 (*VAL/VAL*) were significantly higher (100.5 μmol/L), than in individuals with other polymorphisms ($p < 0.001$) [21].

Among patients with type 1 DM, heterozygous carriers of the *GSTP1* polymorphism (*ILE/VAL*) had higher levels of MDA, GST, and catalase compared to homozygous carriers of the gene polymorphism ($p < 0.001$, $p < 0.001$, $p < 0.05$).

Comparison of the data obtained between patients with type 1 DM, patients with type 2 DM, and the controls revealed, quite naturally, a significant predominance of activity of the AOD enzymes and MDA levels in individuals with DM (Table 3).

DISCUSSION

The study conducted makes it possible to conclude that the abundance of heterozygous *GSTP1* polymorphism (*ILE/VAL*) in individuals with type 1 and 2 DM is significantly higher than that in controls. The genotype distributions for both polymorphisms were compliant with the Hardy–Weinberg equilibrium and showed no significant differences from the data of the SNP database [22]. Furthermore, the rare Val/Val genotype was revealed in individuals with type 2 DM, which was found in none of the surveyed patients with type 1 DM and controls ($\chi^2 = 6.572$, $p = 0.039$).

Patients with type 2 DM, carriers of the rare *GSTP1* polymorphic variant homozygous for allele 2 (*VAL/VAL*), had higher levels of MDA (free radical oxidation markers) and higher activity of the oxidative stress enzymes. One of the studies has shown a clear correlation between this polymorphic variant and the development of type 2 DM, however, the impact of this variant on the development of such complication, as diabetic polyneuropathy, has not been proven [23]. In our study, patients with the rare homozygote for allele 2 had a significantly more severe course of DN (average GFR — 48 mL/min/1.73 m^2; average 24 h urinary albumin excretion rate — 0.9 g/L). Perhaps, this is due to the fact that this specific genetic variant causes the decrease in activity of the protein produced (GST), which does not ensure adequate detoxification of xenobiotics and results in the increase in activity of free radical oxidation. This is reflected in the increase in the free radical oxidation marker (MDA) levels. Thus, conditions are being created for active lipid peroxidation in all cells, especially in the cells most sensitive to hypoxic damage, the nephrons. The multifaceted damage to the nephron structure results in the decrease in the renal functions of filtration (elevated creatinine levels and reduced GFR) and reabsorption (elevated 24 h urinary albumin excretion rate) in the cohort of patients with type 2 DM. Similar data were obtained in the study, during which the researchers confirmed the correlation between the rare homozygous polymorphism (VAL/VAL) and the end-stage kidney disease [24].

Heterozygous (*ILE/VAL*) carriers of the *GSTP1* (*I105V*) gene with type 1 DM had significantly higher activity of the AOD enzymes and MDA levels compared to the homozygous polymorphisms. GST, the antioxidant enzyme, protects the tissues against oxidative damage typical for many health conditions, especially those like type 1 DM and its chronic complications. This polymorphism is likely to be more pathogenic, when it is in heterozygous state. Similar results were obtained in the study that revealed the correlation between this genetic polymorphism and the development of the diabetic complication (cardiovascular autonomic neuropathy) in patients with type 1 DM [25]. Patients with type 1 DM and the heterozygous polymorphism variant also demonstrate a significant increase in the levels of triglycerides (1.6-fold). Based on these data, we can assume that heterozygous carriers have the reduced triacylglycerol lipase activity, which results in slower breakdown of triglycerides. This is associated with the influence of oxidative stress and some hormones, such as norepinephrine, epinephrine, glucagon, etc. it has been reported that activity of this enzyme can change under the influence of the discussed factors [26]. Furthermore, significantly higher glycated hemoglobin levels were determined in this subgroup of patients, which also correlated with elevated MDA levels and increased activity of CAT and GST.

CONCLUSIONS

The personalized medicine is based on the tailored approach to the characteristics of each patient. Introduction of the *GSTP1* (*Ile/Val*) gene molecular genetic assessment into clinical laboratory diagnosis will make it possible to identify patients

with type 1 and 2 DM having high levels of oxidative stress and increased risk of severe DN. When performing clinical laboratory diagnosis, it is recommended to determine the heterozygous (*ILE/VAL*) variant of *GSTP1* (*I105V*) in individuals with type 1 DM, which is associated with the increased free radical oxidation and activity of the AOD enzymes and can result in the significantly higher glycated hemoglobin and triglyceride levels. As for patients with type 2 DM, it is recommended to determine the rare *GSTP1* (*I105V*) gene variant homozygous for allele 2 (*VAL/VAL*) that is associated with the increased free radical oxidation and activity of the oxidative stress enzymes, as well as with the reduced renal function (increased 24 h urinary albumin excretion rate and reduced GFR). Such studies can provide the basis for development of the genetic panel, in which the polygenic sequences that improve reliability and ensure more accurate prediction of the severity and timing of the onset of diabetic complications will be determined. However, this requires expansion of the research in this field.

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THE IMPACT OF BACKGROUND LYMPHOPENIA ON THE REACTIVITY OF NONSPECIFIC IMMUNITY IN RESPONSE TO TOTAL BODY COLD EXPOSURE

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Lymphopenia is a condition in which there are lower than normal counts of lymphocytes in the blood. Combination of lymphopenia and prolonged exposure to low temperatures leads to a reduction of adaptive resources, increasing risks of chronic inflammatory processes and secondary environmentally induced immunodeficiencies. The aim of the study was to compare characteristics of immune reactivity in response to cold exposure depending on background level of lymphocytes. Changes in hematologic and immunologic parameters in 203 participants before and immediately after short-term cold exposure were studied. Measurements included skin temperature (forehead, backside of palm), blood pressure, heart rate, leukogram, and hemogram. Levels of ferritin, lactoferrin, transferrin, interleukin-6, interleukin-1β, TNFα, erythropoietin, and irisin were determined using the enzyme immunoassay method. Apoptosis and necrosis of lymphocytes were assessed by flow cytometry analysis using AnV/PI double staining assay. Regardless of the background level of lymphocytes in peripheral blood, same-type responses to short-term cold exposure were observed in cardiovascular system as well as in irisin and ferritin levels, providing an evidence of activating thermoregulation and thermal homeostasis mechanisms. Lymphopenia is associated with a decrease in activity of nonspecific defense - in response to cold exposure there were no changes in level and functional activity of circulating neutrophil granulocytes that can increase the risks of chronicization of infectious processes in this group.

Keywords: lymphopenia, adaptation, human, NLR, ferritin, transferrin, lactoferrin, cold exposure

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ВЛИЯНИЕ ФОНОВОЙ ЛИМФОПЕНИИ НА РЕАКТИВНОСТЬ ПОКАЗАТЕЛЕЙ НЕСПЕЦИФИЧЕСКОГО ИММУНИТЕТА В ОТВЕТ НА ОБЩЕЕ ХОЛОДОВОЕ ВОЗДЕЙСТВИЕ

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Лимфопения — состояние, при котором концентрация лимфоцитов ниже физиологической нормы. Сочетание лимфопении и длительного воздействия низких температур приводит к сокращению резервов адаптационных ресурсов, повышая риск формирования хронических воспалительных процессов и вторичных экологически обусловленных иммунодефицитов. Цель исследования — сравнить особенности реактивности иммунных показателей в ответ на общее охлаждение в зависимости от фонового уровня лимфоцитов. Проведено изучение изменения гематологических и иммунологических показателей у 203 человек до и сразу после общего охлаждения. У обследованных проводили измерение температуры лба и тыльной стороны ладони, артериального давления и частоты сердечных сокращений, лейкограмму и гемограмму. Методом иммуноферментного анализа определено содержание ферритина, лактоферрина, трансферрина, интерлейкина-6, интерлейкина-1β и TNFα, эритропоэтина, ирисина. Уровень апоптоза и некроза лимфоцитов определяли методом проточной цитометрии двойным окрашиванием AnV/PI. Вне зависимости от фонового уровня лимфоцитов в периферической крови регистрировали однотипные реакции на общее кратковременное охлаждение со стороны сердечно-сосудистой системы, уровня ирисина и ферритина, что свидетельствует о включении механизмов терморегуляции и сохранении теплового гомеостаза. Лимфопения ассоциируется со снижением активности неспецифической защиты, в ответ на холодовое воздействие не происходит изменения уровня и функциональной активности циркулирующих нейтрофильных гранулоцитов, что повышает риск хронизации инфекционных процессов в данной группе.

Ключевые слова: лимфопения, адаптация, человек, NLR, ферритин, трансферрин, лактоферрин, холод

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Соблюдение этических стандартов: исследование проводили с письменного согласия волонтеров, в соответствии с принципами Хельсинкской декларации 1975 г. (2013 г.). Исследование одобрено этической комиссией ИФПА ФГБУН ФИЦКИА УрО РАН (протокол № 4 от 7 декабря 2016 г., протокол № 6 от 14 февраля 2022 г.).

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Described mechanisms of lymphopenia include impaired maturation and differentiation of lymphocytes, inhibition of lymphocyte release from lymphoid tissues, enhancement of lymphocyte migration into tissues as well as death of lymphocytes with increased sensitivity to complement-mediated cytolysis, activation of apoptosis and necrosis. Living in the North requires special adaptations to low temperatures resulting in a decrease in organism's reserve abilities. Cold exposure affects the thymus that is manifested as thymus hypotrophy, reducing lymphocyte abundance and increased intensity of apoptosis, which is further recorded as lymphopenia in peripheral blood [1, 2]. Cold stress leads to depletion of lymphoid tissue of mucous membranes with increasing degenerative processes thus reducing the effectiveness of defense at the "entrance gate" of infection [3, 4]. Decreased activity of cellular and humoral reactions in northern inhabitants is manifested in a higher frequency of acute and chronic infectious diseases, allergies, autoimmune processes and malignancies [5–7]. In pathological state, lymphopenia is accompanied by high levels of proinflammatory cytokines IL-6 and TNFα leading to activation of lymphocyte apoptosis and forming a destructive positive feedback loop [8]. Asymptomatic lymphopenia is often detected in people living in environmentally adverse areas and extreme climatic conditions; in the North, during periods of minimum daylight hours, the reported frequency of lymphopenia in working-age adults is up to 19.86% [9–11]. Adverse climatic effects exert stress on the body and impare the immune system. Prolonged decrease in the number of functionally active lymphocytes providing protective immune reactions significantly increases the risk of severe infectious diseases and their transition to chronic forms. In response to cold exposure, metabolic activity changes most rapidly with an increase in such biochemical parameters as concentrations of free fatty acids, C-reactive protein, glucose, etc. [12]. Factors of innate immunity are most resistant to the influence of cold exposure while for lymphocytes, glucose is a necessary substrate to increase their energy supply and active functioning. The aim of the study was to compare the characteristics of immune reactivity in response to total body cold exposure depending on background level of lymphocytes.

METHODS

Hematological and immunological parameters were measured before and immediately after total body cold exposure in two groups of volunteers (203 participants in total) depending on a background level of peripheral blood lymphocytes. The study included practically healthy individuals of working age who had no acute diseases or exacerbation of chronic diseases during the study period as well as previously and/or currently not engaged in hardening. Persons of working age who had acute chronic diseases and their exacerbations during the study period as well as previously or currently engaged in hardening were excluded accordingly. In the trial, the participants spent 5 minutes in USHZ-25N cold chamber (Xiron-Kholod; Russia) at –25 °C in cotton clothes under constant video monitoring. The first group of participants had a background lymphopenia (n = 70, including 59 women and 11 men; lymphocyte count 1.26 (1.09–1.37) × 10^9/L). The second group included participants having a normal lymphocyte count (n = 133, including 94 women and 39 men; with lymphocyte count 2,08 (1,81–2,45) × 10^9/L (p^{1-2} < 0,0001)). Skin temperatures at forehead and backside of palm, blood pressure and heart rate were measured before and immediately after the cold exposure. Blood samples were collected by qualified staff before and immediately after staying in cold chamber, from the ulnar vein using Vaccuette Blood Collection tubes (with EDTA for plasma and hematologic parameters; with clotting activator for getting serum). Serum and plasma were separated by centrifugation. The samples were frozen once at –20 °C. Hemograms and leukograms were determined using Automated Hematology Analyzer XS-500i (Sysmex; Japan). Ferritin (ORGENTEC Diagnostika; Germany), lactoferrin (HycultBiotech; USA), transferrin (AssayPro; USA), IL6, IL1β and TNFα (Bender MedSystems; Austria), erythropoietin (Vector Best; Russia), irisin (BioVendor; Czech Republic) levels were measured and analyzed using Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific; Finland). The amounts of lymphocytes undergoing apoptosis or necrosis were determined by flow cytometry on Epics XL flow cytometer analyzer (Beckman Coulter; USA) by double staining with annexin-V (AnV) and propidium iodide (PI), counting at least 5000 cells. The results were evaluated by cell staining: live cells (AnV–/PI–), apoptosis (AnV+/PI–), necrosis (AnV–/PI+). Statistical analysis was carried out using Statistica 6.0 software package (StatSoft; USA). Shapiro–Wilk test was used for testing the normality of data. The data were presented as mean (M) ± standard deviation (SD) values. If the distribution was close to normal, t-test was used to compare the results; differences were considered significant at $p < 0.05$. If the distribution differed from normal, data were presented as median (Me) and 25–75% quartiles. Statistical significance of differences was assessed using the non-parametric Mann-Whitney test. The critical level of significance (p) for testing statistical hypotheses was assumed to be 0.05.

RESULTS

The individuals with lymphopenia had a decreased level of neutrophils in peripheral blood: 2.44 (1.93–2.93) × 10^9 /L, with 38.57 ± 2.29% frequency of neutropenia. In individuals with physiologic lymphocyte levels, higher levels of neutrophil granulocytes were observed (3.02 (2.33–3.64) × 10^9/L ($p < 0.001$), neutropenia was detected in 15.79 ± 1.88% of cases. Low count of neutrophil granulocytes is associated with a decrease in their phagocytic activity. Thus, the rate of active phagocytes amounted to 68.53% in the first group (lymphopenia) while in practically healthy participants is was 72.25%. The neutrophil-to-lymphocyte ratio (NLR) was found to be 2.11 in group 1 and 1.49 in group 2. NLR is an important marker of conditions accompanied by systemic inflammation. Elevated NLR is known to be associated with infections, stroke, heart attack, cancer, autoimmune diseases, tissue damage and higher risk of morbidity [13–17]. In both groups, NLR was less than 3.0 which is normal. However, the higher NLR values in the group with lymphopenia evidenced imbalance of immune pathways of inflammation and can be considered as a criterion of increased systemic inflammation risks.

Assessement of apoptosis (AnV+/PI–) and lymphocyte necrosis (AnV–/PI+) showed that necrotic cells counts did not differ significantly in two groups: 0.74 % AnV–/PI+ lymphocytes in group 1, and 0.67% in group 2. The number of lymphocytes labeled for apoptosis was higher in individuals with normal lymphocyte levels in peripheral blood (5.43%), individuals with lymphopenia had 3.68% AnV+/PI– lymphocytes ($p < 0.01$). Thus, lymphopenia in this case is not associated with increased levels of cell death — it is rather a variant of compensatory adaptive reaction, and the impact of adverse factors leads to a shift of parameters out of normal physiological range.

In lymphopenia hemograms, we observed lower counts of erythrocytes (4.41 (4.08–4.73) and 4.68 (4.31–4.99) × 10^6 /L,

Table. Levels of iron-containing proteins in peripheral blood serum, M ± *m*, $p < 0.01$

	Ferritin, ng/mL	Lactoferrin, ng/mL	Transferrin, ug/mL
Group 1 (Lymphopenia)	43.91 (23.22–53.55)	394.85 (180.24–383.92)	827.35 (360.30–515.90)
Group 2 (Normal lymphocyte levels)	63.90 (24.38–87.21)	334.71 (169.80–470.80)	473.56 (351.40–549.6)

respectively, $p < 0.001$) and hemoglobin (127, 70 (118.00–138.00) and 137.19 (128.00–149) g/L, respectively, $p < 0.0001$) with no significant differences in mean hemoglobin concentrations in erythrocytes (340.33 (331.00–351.00) and 340.71 (332.00–349.00) g/L). The frequency of detection of less than 120 g/L hemoglobin concentration was 30.75 ± 2.15% in the first group and 16.67 ± 1.46% in the second group. Less than 4×10^6 erythrocytes/L values were actually four times more often detected in lymphopenia (in 20.51% and 6.06% of the participants, respectively). No significant differences in erythropoietin concentrations were observed in the two groups; it was 30.02 (13.25–35.48) mMe/mL in individuals with low lymphocyte counts and 29.68 (17.31–37.11) mMe/mL in individuals with normal lymphocyte counts.

Absorption and accumulation of iron play an important role in regulation of erythropoiesis as well as in adaptation to cold. Ferritin can serve as an indirect marker for total body iron store. Transcription of the ferritin H isoforms mRNA and accumulation of ferritin were enhanced by cold acclimation [18]. Levels of this iron-containing protein in lymphopenia occurred to be within the physiological norm with a tendency to lower concentrations as compared with individuals with normal lymphocyte counts (Table). No statistically significant differences in lactoferrin levels were found for the two groups. Lymphopenia is associated with the almost 2-fold higher transferrin levels in peripheral blood. Hypoxia and low temperatures are factors enhancing the expression of the transferrin gene and, consequently, transferrin blood level. High levels of transferrin, on the one hand, increases iron supply to tissues to compensate for oxygen deficiency but on the other hand, transferrin promotes the activation of thrombin which increases the risk of hypercoagulability and as a consequence, thromboembolic and cardiovascular pathologies [19, 20].

In both groups, levels of cytokines in peripheral blood were within the physiologic norm, no significant differences were found. In case of asymptomatic lymphopenia, IL6 levels were 2.48 ± 0.41 pg/mL in group 1 and 3.74 ± 0.35 pg/mL in group 2, IL1β levels 5.01 ± 0.61 and 4.46 ± 0.67 pg/mL, and TNFα levels 6.32 ± 1.03 and 7.32 ± 0.91 pg/mL, respectively.

After the cold exposure, an adaptive response of the cardiovascular system was recorded in both groups, with a tendency to increase blood pressure and decrease heart rate (Figure).

In both groups, a significant decrease was observed in forehead skin temperatures (from 36.6 ± 0.06 to 34.05 ± 0.45 °C in group 1, $p < 0.0001$; from 36.4 ± 0.10 to 33.78 ± 0.32 °C, in group 2, $p < 0.0001$) as well as in back of the palm skin temperatures (from 33.2 ± 0.33 to 32.4 ± 0.24 °C in group 1, $p < 0.05$; from 33.61 ± 0.36 to 32.54 ± 0.23 °C in group 2, $p < 0.05$). Total body short-term cold exposure was accompanied by an increase in lymphocyte levels in individuals with lymphopenia up to 1.32 (1.13–1.48) × 10^9 /L ($p < 0.05$), with no significant change in the "control" group: 2.02 (1.76–2.35) × 10^9 /L. For neutrophil granulocytes, the opposite response was observed. In group 2 (normal lymphocyte levels) concentration of neutrophils increased by 11% up to 3.27 (2.57–4.01) × 10^9/L ($p < 0.05$) while in group 1 (lymphopenia) no changes in neutrophil content were observed. In both groups there was a decrease in the level of irisin (from 4.25 (1.81–6.70) to 3.52 (1.30–5.75) μg/mL in group 1; from 2.99 (1.63–7.14) to 2.38 (1.65–5.66) μg/mL in group 2 which may be an evidence of activating mechanisms of non-shivering thermogenesis.

Cold exposure was associated with an increase in ferritin concentration up to 57.67 (41.67–65.10) ng/mL($p < 0.01$) in group 1 (lymphopenia) and up to 76.46 (25.29–98.29) ng/mL ($p < 0.01$) in group 2 (normal lymphocyte level); lactoferrin content increased in group 2 only (up to 498.85 (124.68–485.97) ($p < 0.0001$); transferrin content did not change in both groups — 558.60 (421.70–940.70) mg/dL in group 1 and 423.30 (351.40–549.60) mg/dL in group 2).

DISCUSSION

Adaptive abilities depend on the levels of reactivity and resistance providing stress reaction or training reaction in response to environmental factors. Lymphopenia is known to be a negative prognostic marker in various pathological conditions, and its combination with the need to adapt to low temperatures leads to overstress of regulatory systems and failure of adaptation [21–23]. Asymptomatic lymphopenia commonly found in people living in the North is accompanied by neutropenia and lower activity of phagocytic defense.

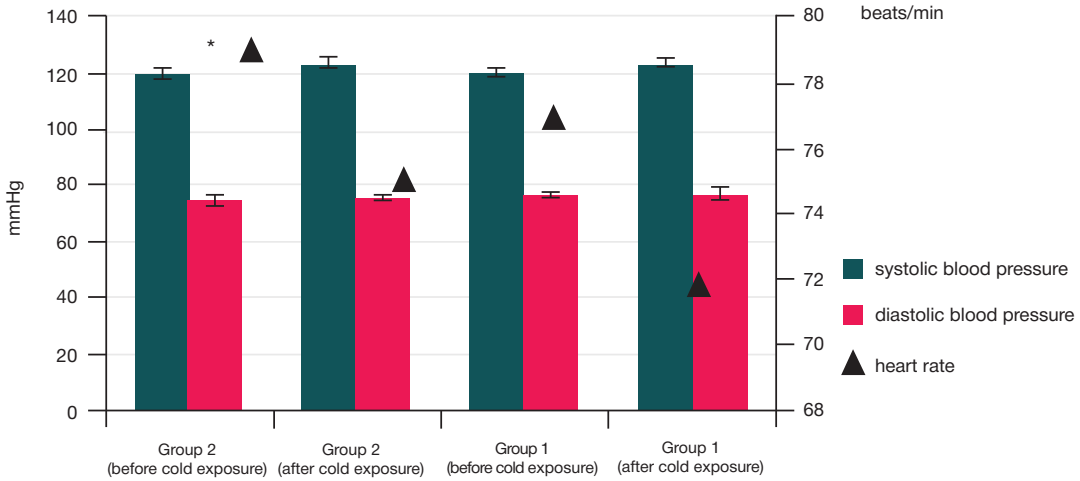

Fig. Changes in blood pressure and heart rate after short-term cold exposure. * — $p < 0.01$

Redistribution of leukocytes and increased transfer of neutrophil granulocytes to tissues under the influence of adverse climatic factors plays a role in the etiology of neutropenia in northerners [24, 25]. Low lymphocytes counts in peripheral blood are associated with tissue hypoxia which may be a consequence of abnormal morphofunctional state of erythrocytes caused by exposure to low temperatures and oxidative stress [26–28].

In addition, people living in the North are characterized by structural changes in erythrocyte membranes with increased membrane viscosity, and as a consequence, a decrease in the rate of gas diffusion and oxygen supply to tissues [29–31]. NLR is shown to be higher in individuals with lymphopenia as compared with individuals having normal lymphocyte levels. Combination of high NLR with insufficient oxygen supply is an adverse marker in patients with infectious inflammatory diseases [32]. In response to cold exposure, similar cardiovascular reactions have been observed in both groups regardless of the background level of lymphocytes, that are manifested in increased blood pressure and decreased heart rate. Decreased level of irisin, a protein involved in metabolism and thermoregulation is an evidence of activating thermoregulatory mechanisms [33, 34]. Irisin upregulates expression of uncoupling protein 1 (UCP1) and leads to non-shivering thermogenesis and increased heat production. No changes in lactoferrin concentration have been found in individuals with background lymphopenia after the short-term cold exposure. An increase in the concentration of this protein is associated with degranulation of neutrophils and reflects the level of their activation. Thus, lymphopenia combined with neutropenia and decreased functional activity of neutrophil granulocytes significantly increases the risk of adaptive failure, and systematic exposure to cold increases the probability of chronicization of infectious diseases. In addition, a high level of transferrin persisting after cold exposure increases risk of hypercoagulability, thrombosis and cardiovascular events. In both groups, actually the same increase in ferritin levels was observed (in + 21.10% in group 1 and + 19.96% in group 2) which may be an evidence of thermal homeostasis since the induction of ferritin heavy chain expression promotes survival in cold environments by detoxifying iron forms that generate reactive oxygen species [35].

CONCLUSIONS

Asymptomatic background lymphopenia is associated with insufficient oxygen supply and higher levels of neutropenia. Regardless of the background level of lymphocytes in peripheral blood, same-type responses to short-term cold exposure are observed in cardiovascular system as well as in irisin and ferritin levels, providing an evidence of activating thermoregulation mechanisms. Cold exposure did not induced activation of nonspecific defense and did not change level and functional activity of circulating neutrophil granulocytes that can increase risks of chronicization of infectious processes in this group. The obtained data can be used in monitoring related to environmental physiology, for developing methods for assessing risks of maladaptive reactions to cold exposure and correcting immunity disorders in people living in the North.

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ANTIOXIDANT EFFECTS OF THE SYNTHETIC THYRONAMINE ANALOGUE IN EXPERIMENTAL CEREBRAL ISCHEMIA

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The oxidative stress associated with ischemic stroke is a major factor damaging the nervous tissue. Thyroid hormones have a significant effect on the body's redox status, however, the impact of their derivatives, thyronamines, considered as potential neuroprotectors, on the characteristics of lipid peroxidation (LP) is not clearly understood. The study was aimed to assess the impact of the T0AM thyronamine synthetic analogue on the main LP indicators in the model of acute cerebral ischemia. Permanent ligation of the right common carotid artery was performed to simulate acute cerebral ischemia in white rats. The animals were divided into two groups: the control group receiving no treatment and the experimental group, to which the T0AM thyronamine synthetic analogue was intraperitoneally administrated (75 mg/kg of the rat's body weight). After 24 h the rat was decapitated, and the cerebral cortex tissue was extracted for biochemical analysis. The following LP indicators were determined by spectrophotometry: malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx). When administering the T0AM thyronamine synthetic analogue, a significant (2-fold) decrease in MDA levels was observed in the ischemic hemisphere ($p = 0.022$), along with the 2.49-fold increase in the GPx activity in the brain tissue ($p = 0.004$) of the intact hemisphere and the 2.65-fold increase in its activity ($p = 0.021$) in the ischemic hemisphere, as well as the 1.23-fold increase in SOD activity in the ischemic hemisphere ($p = 0.042$). The T0AM thyronamine synthetic analogue has a great potential in terms of activation of the antioxidant protection mechanisms in the cerebral cortex of white laboratory rats under conditions of acute hemispheric ischemia.

Keywords: thyronamines, antioxidants, neuroprotection, ischemic stroke, oxidative stress, lipid peroxidation

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АНТИОКСИДАНТНЫЕ ЭФФЕКТЫ СИНТЕТИЧЕСКОГО АНАЛОГА ТИРОНАМИНА ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ИШЕМИИ ГОЛОВНОГО МОЗГА

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Окислительный стресс при ишемическом инсульте — один из основных факторов, повреждающих нервную ткань. Тиреоидные гормоны оказывают существенное влияние на редокс-статус организма, однако влияние их производных, тиронаминов, рассматриваемых в качестве потенциальных нейропротекторов, на показатели перекисного окисления липидов (ПОЛ) изучено недостаточно. Целью исследования было изучить влияние синтетического аналога тиронамина T0AM на основные показатели ПОЛ в модели острой ишемии головного мозга. Для моделирования острой ишемии головного мозга у белых крыс выполняли необратимую перевязку правой общей сонной артерии. Животные были разделены на две группы — контрольную без лечения и экспериментальную, в которой интраперитонеально вводили синтетический аналог тиронамина T0AM (75 мг/кг массы тела крысы). Спустя сутки крысу подвергали декапитации, и ткань коры больших полушарий головного мозга извлекали для биохимического анализа. Из показателей ПОЛ определяли малоновый диальдегид (МДА), супероксиддисмутазу (СОД), глутатионпероксидазу (ГПО) спектрофотометрически. На фоне введения синтетического аналога тиронамина T0AM наблюдали статистически значимое снижение содержания МДА в ишемизированном полушарии в 2 раза ($p = 0,022$), повышение активности ГПО в ткани головного мозга в 2,49 раза ($p = 0,004$) для интактного и в 2,65 раза ($p = 0,021$) — для ишемизированного полушарий и увеличение активности СОД в ишемизированном полушарии в 1,23 раза ($p = 0,042$). Синтетический аналог тиронамина T0AM обладает значительным потенциалом в отношении активации механизмов антиоксидантной защиты в коре головного мозга белых лабораторных крыс в условиях острой полушарной ишемии.

Ключевые слова: тиронамины, антиоксиданты, нейропротекция, ишемический инсульт, окислительный стресс, перекисное окисление липидов

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Stroke is one of the leading causes of mortality all over the world and the major cause of permanent disability that exerts a heavy economic burden on the entire society. The development of ischemia is associated with the rapid death of millions of neurons within seconds. Unfortunately, today there are still no effective neuroprotective agents capable of mitigating this process [1].

Oxidative stress (OS) accompanied by the release of reactive oxygen species (ROS) is one of the main mechanisms underlying such damage. The brain is particularly sensitive to oxidative damage, since it contains large amounts of polyunsaturated fatty acids representing one of the prime targets for ROS. In the rodent experiment, high levels of lipid peroxidation (LP), the products of which activate phospholipase A2 ensuring cleavage of the cell membrane phospholipids with the release of pro-inflammatory mediators, are observed in the ischemic stroke (IS) focus 24 h after the permanent middle cerebral artery (MCA) occlusion. Low levels of antioxidants with subsequent antioxidant buildup are reported in the same time period [2, 3]. The DNA bases are also to the great extent susceptible to the damaging effects of oxidants. Consequently, mutations and deletions occur in both primary and secondary structure of both nuclear and mitochondrial DNA, and the latter is more vulnerable, since it is located closer to the original source of ROS and has a lower repair capacity compared to the nuclear DNA. The LP products can behave as triggers of the p53 signaling pathway, causing changes in the membrane structure and the loss of mitochondrial DNA function [4]. Disruption of redox homeostasis is associated with damage to the nervous system that can result in the autoimmune and neurodegenerative diseases. In terms of OS intensity assessment, such indicators, as malondialdehyde (MDA) being one of the OS biomarkers, endogenous antioxidant superoxide dismutase (SOD), and glutathione peroxidase (GPx) that catalyzes the reduced glutathione oxidation, are of special interest.

The contribution of thyroid hormones (TH) to maintaining the redox status is ambiguous. There are literature data suggesting that hyperthyroidism results in the increased ROS production, while hypothyroidism leads to the decrease in ROS production, thereby reducing the antioxidant activity [5, 6]. The neuronal mitochondria represent one of the targets for both TH and their derivatives (particularly thyronamines), and morphofunctional alterations in the functioning of these organoids associated with the TH deficiency are observed. Upregulation of the genes, responsible for mitochondrial palmitate beta-oxidation affected by triiodothyronine resulting in the increase of adenosine triphosphate (ATP) essential for normal function of the ion pumps, was demonstrated in the astrocyte culture [7, 8]. Considering the central role of astrocytes in protecting neurons in ischemic brain damage, the authors concluded that reduction in the lesion size in experimental transient ischemia resulted specifically from normalization of energy exchange in astroglia ensured by T3.

However, this is just one of the possible mechanisms underlying neuroprotection ensured by TH. The description of the role of their derivatives, thyronamines, in protection of neurons, is usually limited to the hypothermic effect reported in the literature. We have assumed that thyronamines, specifically T0AM, also can contribute to the nervous tissue antioxidant protection. The study was aimed to assess the concentrations of products actively reacting with thiobarbituric acid (TBA-AP), SOD, and GPx in the brain tissue of laboratory rats after experimental acute ischemia against the background of administration of the T0AM thyronamine synthetic analogue (SA-T0AM) as a proposed neuroprotector.

METHODS

The authors synthesized the T0AM thyronamine synthetic analogue, 4-[4-(2-aminoethoxy)benzyl]aniline hydrochloride, by the method reported in the literature [9]. The structure of the resulting compound was confirmed by the ^{1}H and ^{13}C NMR spectroscopy.

A total of 40 male and female animals with the body weight of 190–210 g from the vivarium of the Gusak Institute of Emergency and Reconstructive Surgery were selected for the experiment. Permanent ligation of the right common carotid artery (CCA) in white non-linear laboratory rats was selected as a model of acute cerebral ischemia. According to the published studies, this model causes small cortical infarcts [10]. Surgery was performed under general anaesthesia (Calypsol, 100 mg/kg of the rat's weight). Dimethyl sulfoxide (DMSO) was used as a solvent for the SA-T0AM. In the study focused on assessing biological effects of various solvents, intraperitoneal administration of DMSO to rats in a dose of 5 mL/kg for a month was considered to be relatively safe [11]. The animals were divided into two experimental groups, 20 rats per group. In the first group (Control), the right CCA ligation surgery was performed; 0.5 mL of the DMSO solution + 0.5 mL of the 0.9% NaCl solution were administered intraperitoneally 10 min after the ligation. In the second group (Experiment), the animals were administered 0.5 mL of the DMSO solution + 0.5 mL of the 0.9% NaCl solution + SA-T0AM in a dose of 75 mg/kg of the rat's weight after surgery. The optimal dose of 75 mg/kg was selected based on the maximum hypothermia induction at zero mortality, in accordance with the previously reported study [12]. Given the maximum OS activity observed within minutes after the ischemia induction [13], the agent was administered 10 min after the CCA ligation. The animals were subjected to decapitation and extraction of the brain 24 h after the experiment. The cerebral cortex tissue (tissues of the intact hemisphere and the hemisphere affected by ischemia were used separately) was use to determine the LP indicators.

The weighted portion of the fresh rat cerebral cortex tissue was homogenized for at least 10 min in the glass homogenizer with the 50 mM Tris buffer containing 1 mM of EDTA and 0.25 M of sucrose, pH 7.4, in a ratio of 1:3. The as-prepared homogenate was frozen at a minimum of –70 °C for 24 h. After thawing is was centrifuged for 30 min at 4000 rpm, then the centrifugate was 6-fold diluted (50 µL of supernatant + 250 µL of 5 mM potassium phosphate buffer containing 1×10^{-4} M of EDTA, pH 7.8).

Protein was quantified by the photometric Lowry protein assay at λ = 750 nm in a 5 mm cell. The assay results are required to convert the SOD, GPx, and TBA-AP content in the animal brain homogenate (per 1 mg of protein). The method to determine SOD activity in the brain tissue homogenate is based on the enzyme capability of inhibiting epinephrine autoxidation to adrenochrome at pH 10.2. The oxidation kinetics was measured by spectrophotometry at λ = 480 nm in a 10 mm cell using the Eppendorf EPAC 6140 biochemical analyzer (Eppendorf AG; Germany). The TBA-AP concentration was determined based on the reaction of MDA with 2-thiobarbituric acid (TBA) yielding the colored "trimethine complex", the concentration of which was determined by photometry at λ = 532 nm in a 10 mm cell. Extinction was measured in the Eppendorf EPAC 6140 biochemical analyzer. The GPx activity was estimated based on the changes in the amount of reduced glutathione (GSH) before and after incubation with the model substrate (tert-butyl hydroperoxide) based on the reaction with Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid). Absorbance was measured

Table. The antioxidant protection indicators in the brain tissue of model rats with acute hemispheric ischemia (SOD, GPx and TBA-AP levels are provided per 1 mg of protein)

Groups	Control		Experiment		*p*
Indicator	*n*	Median	*n*	Median	
SOD, U, intact hemisphere	16	60	16	63	0.75
SOD, U, ischemic hemisphere	18	59	18	72.5	0.042
GPx, μmol/min*mg, intact hemisphere	16	278.5	18	693	0.004
GPx, μmol/min*mg, ischemic hemisphere	16	304	18	805	0.021
TBA-AP, μmol, intact hemisphere	20	0.44	20	1.5	< 0.001
TBA-AP, μmol, ischemic hemisphere	20	0.7	20	0.34	0.022

in a cell with the pathlength of 10 mm at 412 nm using the Eppendorf EPAC 6140 biochemical analyzer.

Statistical processing of the data obtained was performed in the R software package (R Core Team, 2018). According to the Shapiro–Wilk test results, the distribution of the TBA-AP ($W = 0.79$, $p = 0.01$), GPx ($W = 0.860$, $p = 0.016$), and SOD ($W = 0.89$, $p = 0.41$) parameters in the hemisphere affected with ischemia was non-normal. The nonparametric Mann–Whitney U test was used to reveal the differences between samples.

RESULTS

The results of biochemical tests characterizing the activity of antioxidant systems in the brain tissues of laboratory animals in the model of acute cerebral ischemia are provided in the Table and the Figure.

Comparison of TBA-AP levels in the intact hemisphere showed that these were 3.4 times higher in rats receiving SA-T0AM than in control animals ($p < 0.001$). However, the

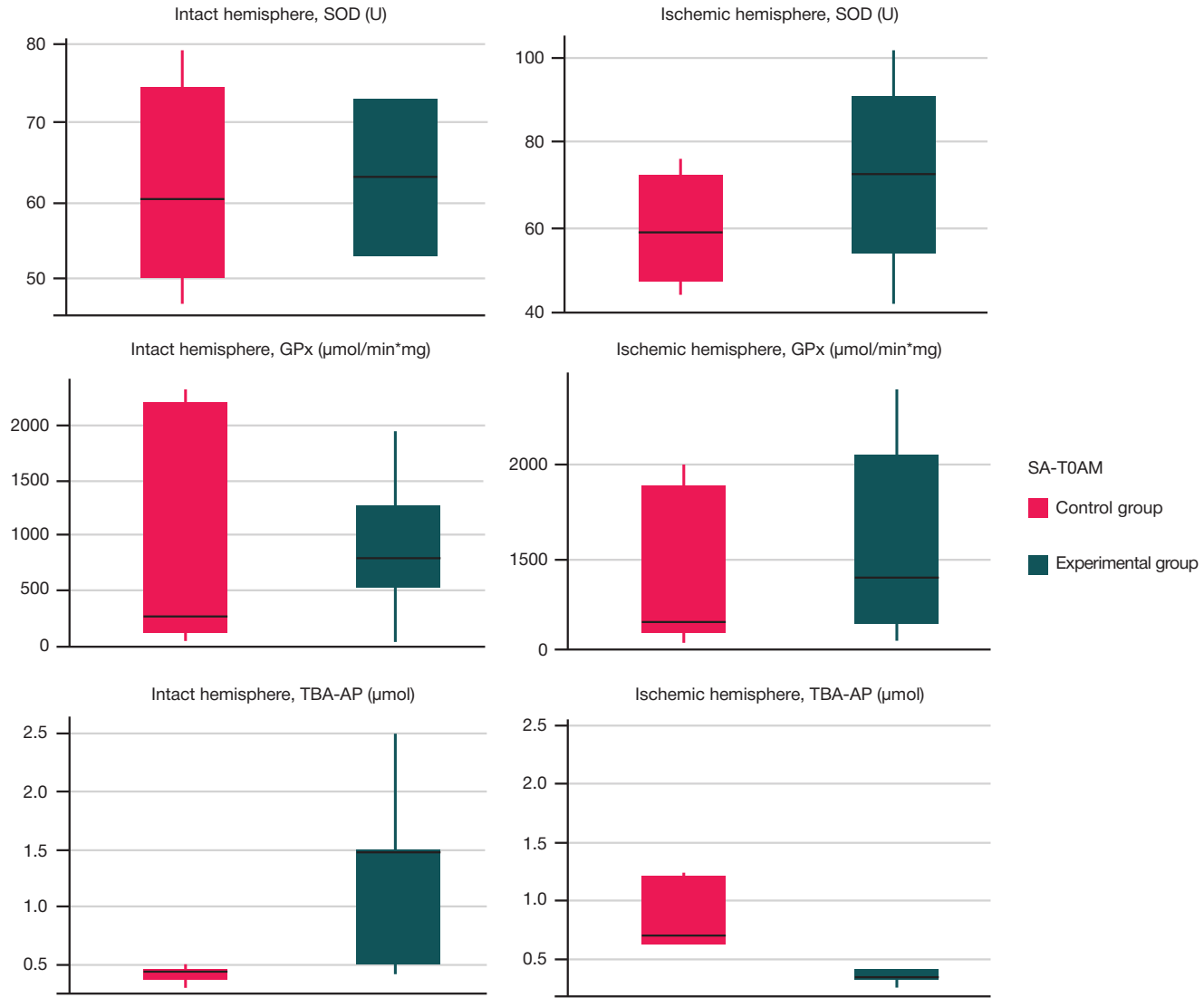

Fig. The oxidative stress marker activity in the brain of model rats with acute hemispheric ischemia. TBA-AP — concentration of products actively reacting with thiobarbituric acid; SOD — superoxide dismutase; GPx — glutathione peroxidase

levels of TBA-AP in the hemisphere affected by ischemia in the rats administered the studied potential neuroprotector were approximately twice lower than in control animals with ischemia receiving no therapy ($p = 0.022$).

Comparison of GPx activity in rats of the control and experimental groups showed that the SA-T0AM administration resulted in the 2.49- and 2.65-fold increase in activity of this enzyme, respectively, in the cortical tissue of both intact hemisphere ($p = 0.040$) and the hemisphere affected by ischemia ($p = 0.021$).

No significant differences in SOD activity in the intact hemisphere cortical tissue between the control and experimental rats were revealed ($p = 0.750$). However, the SOD activity in the hemisphere affected by ischemia in the animals receiving SA-T0AM turned out to be 1.23 times higher than that in control rats ($p = 0.042$).

DISCUSSION

Changes in MDA levels associated with acute ischemia

The increase in the TBA-AP levels observed in the homogenates of the rat brain hemispheres suggests activation of the OS processes in ischemia. MDA is a stable and toxic LP product. The increase in the levels of MDA, the main TBA-AP component, results in disturbance of permeability and subsequent cell membrane disruption, the release of lysosomal enzymes, and activation of the processes underlying lysis of the cellular structures.

The researchers have shown a significant increase in blood levels of MDA in patients with IS, without any correlation with the disease outcome; the other study revealed the relationship between the serum MDA levels and the functional disease outcome after 3 months, and, therefore, suggested to use MDA as a biopredictor [2, 3]. A number of authors confirm that there is a significant positive correlation between the MDA levels and the functional outcome of stroke a week after the patient admission to the unit; one of the studies has shown that the MDA levels determined at admission and 7 days later can be used to predict the patient's functional disability 6 months later based on the mRS scale [14, 15]. A significant correlation between the MDA levels and the stroke severity based on the NIHSS scale has been also revealed. Huge amounts of free radicals accumulate in the penumbra (as indicated by MDA accumulation) due to insufficient oxygen uptake. The extent of damage depends on the activity of the antioxidant protection mechanisms. In case of severe stroke, antioxidants fail to ensure binding of free radicals due to large amounts of damaged tissues. The antioxidant enzymes are induced enzymes, therefore, their transcription and synthesis take time. Thus, the increase in LP due to insufficient stimulation of the antioxidant protection mechanisms takes place in the early phase of stroke, which reflects the stroke volume, and therefore, the stroke severity; MDA serves as a sensitive marker of this process. The decrease in the levels of TBA-AP in the rat brain tissue homogenate against the background of CA-T0AM administration suggests the decrease in the OS degree in this group of animals [16].

Changes in GPx activity associated with acute ischemia

It is believed that GPx has a protective function; it ensures protection against brain damage. The increase in the infarct size and the apoptosis enhancement are observed in the glutathione peroxidase-1 (GPX1)-knockout mice in the experiment involving MCA occlusion with subsequent reperfusion [17, 18]. The increase in caspase-3 activity enhanced with OS was observed in these animals, which also testified in favor of the fact that ROS sensitive to GPX1 play an important role in regulation of apoptosis. The data confirm that GPX1 can effectively interact with both major neuronal death signaling pathways and the mechanisms of post-ischemic inflammation. This allows some authors to consider GPX1 as a promising tool for therapeutic intervention in the processes related to prevention or regulation of post–ischemic brain damage [19].

The GPx overexpression protective effect on the cerebral neurons of rats with focal ischemia was demonstrated. In the experiment involving 62 animals, the MCA occlusion was simulated, and the viral vectors expressing either GPx1/lacZ (experimental group), or lacZ only (control) were introduced in the striata (ischemic foci) by stereotactic injection. It was found that when the vectors were injected 12 h before surgery, the survival rate of neurons was 36% higher compared to that reported for the control group. When the vector was injected 2 h and 5 h after surgery, the survival rate of neurons was 26% and 25% higher, respectively, compared to controls. The fact that the ischemia severity was the same in both groups was confirmed by morphological data. The authors used immunofluorescence staining to demonstrate that GPx overexpression prevented the release of cytochrome from the neuronal mitochondria and limited the nitrogen-mediated damage to these organoids, suppressed Bax and caspase-3 expression, and activated Bcl-2 expression, which suggested GPx involvement in inhibition of the endogenous apoptosis pathway. Endogenous GPx is synthesized in the neurons, and transfer of the gene with the vector enhances GPx production. Astrocytes protect the neurons against OS due to glutathione they contain, and GPx overexpression also contributes to glutathione transformation into an oxidized form after the reaction with ROS. GPx is capable of directly inhibiting some phases of the apoptosis pathway without reduction of the total ROS levels. For example, the increase in Bcl-2 production against the background of GPx overexpression can inhibit the cytochrome c release. The cytosolic cytochrome forms a large part of the apoptosome of vertebrates, which also contains procaspase-9. The caspase-9 activation induces activation of caspase-3 triggering biochemical disruption of the cells. The data suggest that GPx prevents apoptosis at the stage of cytochrome c release, as evidenced by upregulation of Bcl-2 and downregulation of Bax. Injection of the vector carrying the GPx gene 4–6 h after the development of ischemia can prevent the second phase of caspase activation, thereby reducing the neuronal death rate in the ischemic focus. The researchers determine the therapeutic window of 9–11 h for this method of the GPx gene delivery to the disease site [20].

Thus, the increase in GPx activity represents an endogenous protective mechanism ensuring survival of neurons in stroke, and the SA-T0AM administration considerably increases the activity of this enzyme.

Changes in SOD activity associated with acute ischemia

SOD belongs to the major antioxidant enzymes. Minor SOD activation in the experimental hemisphere homogenates after occlusion is typical for ischemic tissues and suggests adaptive rearrangement of the antioxidant protection patterns in response to disturbances of the oxygen delivery to cells. In general, the literature data on the SOD activity in IS are controversial. Thus, a significant decrease in blood levels of SOD was reported in individuals with IS affecting large (not

small!) blood vessels [21], the same results were obtained when assessing SOD in blood serum of 41 patients with acute IS [22]. However, other researchers, in contrast, point to the rapid increase in plasma SOD levels in patients admitted to the unit and explain this phenomenon by the significant increase in the body's levels of free radicals [23]. Thus, today, the data on the SOD activity alterations associated with cerebral ischemia are controversial and require further investigation.

At the molecular level, the increase in the cytosolic cytochrome c levels together with DNA fragmentation was observed in the SOD2 knockout animals. The rats with SOD1 overexpression, in contrast, had low cytosolic cytochrome c levels. The release of cytochrome c results in the ROS production enhancement due to the respiratory chain inhibition. It is believed that ROS also initiate the release of cytochrome c from mitochondria. Thus, a "vicious circle" of cytochrome c release from the neuronal mitochondria in ischemia is formed, eventually resulting in the apoptotic cascade activation [20].

Our data suggest SOD activation in response to the SA-T0AM administration, which confirms the influence of this potential neuroprotector on the antioxidant protection enhancement in the brain tissue in ischemia.

Influence of thyronamines on the antioxidant protection characteristics

The T1AM and T0AM thyronamines are capable of binding to the TAAR1 receptor (Trace Amine-Associated Receptor 1) in the dose-dependent manner, which is accompanied by production of cyclic adenosine monophosphate (cAMP). However, it is currently difficult to credibly claim that TAAR1 is the only endogenous receptor, through which biogenic amines realize their effects. Thus, the increase in cAMP production at the cellular level is inconsistent with the development of hypothermia and the cardiac function decrease. Therefore, either TAAR1 activation is not G-protein-coupled in certain tissues, or thyronamines also can interact with other forms of TAAR [24]. Perhaps, the effects of thyronamines are also mediated by interaction with the receptors other than TAAR. Some authors note intracellular T1AM accumulation, suggesting the existence of intracellular targets for this TH derivative [25]. It is interesting that T0AM sometimes affects O_2 consumption to the greater extent, than T1AM, despite the fact that this thyronamine is less effective for in vivo hypothermia induction [9].

Thyronamines are natural decarboxylated TH derivatives. The *in vivo* administration of thyronamines often causes the effects opposite to that caused by TH, including the decrease in body temperature. Since it is well-known that the mitochondrial energy transduction apparatus is a potential target for TH and their derivatives, an *in vitro* study of the impact of T0AM and T1AM on the O_2 consumption rate and H_2O_2 release by the rat mitochondria was conducted in 2012. The study involved animals with hypothyroidism due to their low levels of endogenous thyronamines. The authors have found that incubation of mitochondrial preparations with thyronamines causes the decrease in the respiratory chain complex III activity, and endogenous T1AM can significantly reduce the O_2 consumption, thereby probably slowing down the rate at which electrons move through the respiratory chain, and enhance production of ROS by the liver mitochondria in rats with hypothyroidism. Furthermore, T1AM is oxidized by monoamine oxidases of the mitochondrial outer membrane due to O_2 that is subsequently reduced to H_2O_2 [26, 27].

The impact of thyronamine on the GPx enzyme activity in the brain is poorly understood and requires further investigation. Activation of the free-radical oxidation processes, the increase in the levels of ROS, as indirectly evidenced by the increase in the TBA-AP levels and SOD activity in our experiment, trigger the redox signaling processes. It is believed that the Nrf2-Keap1-ARE system is the main system responsible for activation of adaptive mechanisms in the cells under the conditions of OS. The Nrf2 nuclear factor is a transcription factor regulating a number of the antioxidant protection genes that act synergistically, ensuring binding of ROS via a cascade of enzymatic reactions. The Nrf2 target genes are involved in neutralization of free radicals, detoxification of xenobiotics, and maintaining the redox potential. Nrf2 is usually found in the cytoplasm and is bound to the Keap1 protein. The OS changes the position of the sulfhydryl groups in the Nrf2-Keap1 complex, causing dissociation and the Nrf2 transfer to the cell nucleus, where Nrf2 binds to the antioxidant response element (ARE) located in the promoter regions of a number of genes encoding the enzymes involved in the glutathione synthesis and metabolism (glutamate-cysteine ligase, glutathione S-transferase, GPx, glutathione reductase) and other enzymes involved in antioxidant protection (SOD, catalase) [28]. This is how activation of transcription of these genes is triggered; this mechanism can explain, why the TBA-AP levels are higher in the intact hemisphere, to the less extent affected by OS, than in the ischemic hemisphere. It has been shown in the animal model that Nrf2 activation can save the penumbra tissue, but not the tissue in the stroke core, while preventive treatment improves the functional outcome within a month. The animals having deletions in the Nrf2 gene become sensitive to the stress factors and susceptible to cerebral ischemia and other neurological disorders [29]. The increase in GPx activity in response to the increase in TBA-AP levels in the ischemic hemisphere homogenates before and especially after administration of SA-T0AM can be also caused by activation of the Nrf2-Keap1-ARE redox signaling [30].

In other words, the impact of TH and their derivatives (specifically thyronamines) on the redox status of the body and certain body tissues has been confirmed by a number of authors, however, the nature of this impact is extremely controversial and requires further investigation.

CONCLUSIONS

According to our findings, the TBA-AP levels in the white rat's ischemic hemisphere decrease in response to administration of the T0AM synthetic analogue (75 mg/kg of the rat's body weight, intraperitoneally). At the same time, the increase in SOD activity in the ischemic hemisphere along with the significant increase in GPx activity in the tissues of both brain hemispheres is observed in rats of the experimental group. This suggests that SA-T0AM used as a neuroprotector has a great potential in terms of activation of the antioxidant protection mechanisms in the cerebral cortex of white laboratory rats with acute hemispheric ischemia.

In the future we plan to continue the search for the most promising thyronamine synthetic analogues (water-soluble forms with the more prominent hypothermic effect that are more convenient to use in clinical settings), determine their biological properties in the model of focal cerebral ischemia, and identify the signaling pathways, through which their neuroprotective effects are realized.

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IMPACT OF PERSISTENT COLD STRESS ON SPECTRAL CHARACTERISTICS OF EEG ALPHA AND THETA RHYTHMS IN MILITARY ACADEMY CADETS

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The study was focused on the features of spectral characteristics of the EEG alpha and theta ranges in the military academy cadets undergoing specific training to improve cold resistance (cold exposure training). The study was aimed to assess the impact of the military academy cadets' incremental exposure to the graduated cold stress (cold exposure training) on spectral characteristics of the EEG alpha and theta rhythms. Students of the civil higher educational institution and military academy cadets were assessed (58 individuals in total). Cadets underwent a specific program focused on improving cold resistance (cold exposure training). Background EEG in the alpha and theta frequency ranges was recorded. Statistical data processing involved nonparametric comparison using the Mann–Whitney U test. The results were obtained suggesting that there were significant differences in spectral characteristics of alpha and theta rhythms between the group of foreign cadets and the controls. The cold exposure training program was effective in the group of Russian cadets, which had an effect on the cerebral homeostasis stability with some degree of instability of neurodynamic processes in the CNS. In foreign cadets, regular cold exposure training resulted in the pronounced disintegration of cortical-subcortical and intracortical interactions, as well as in the formation of binary alpha-theta structure of background EEG.

Keywords: EEG, alpha rhythm, theta rhythm, adaptation, cold resistance of the body, military cadets

Author contribution: Tolstoguzov SN — research procedure, data analysis, manuscript writing; Fisher TA — data analysis, manuscript writing; Naida YuV — research procedure; Lepunova ON — data analysis.

Compliance with the ethical standards: the study was approved by the Ethics Commitee of the University of Tyumen (protocol № 11 dated 19 February 2024). All patients submitted the informed consent to stady participation.

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ВЛИЯНИЕ СИСТЕМАТИЧЕСКОЙ ХОЛОДОВОЙ НАГРУЗКИ НА СПЕКТРАЛЬНЫЕ ХАРАКТЕРИСТИКИ ТЕТА- И АЛЬФА-РИТМОВ ЭЭГ КУРСАНТОВ ВОЕННОГО ВУЗА

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В настоящем исследовании рассмотрены особенности спектральных характеристик ЭЭГ в альфа- и тета-диапазонах у курсантов военного вуза, проходящих специальную подготовку по повышению холодовой устойчивости (закаливание). Целью работы было изучить влияние поэтапного воздействия дозированных низкотемпературных нагрузок (закаливания) курсантов военного вуза на спектральные характеристики ЭЭГ в альфа- и тета-диапазоне. Исследованы студенты гражданского вуза и курсанты военного вуза (всего 58 человек). Курсанты проходили специальную программу повышения холодовой устойчивости (закаливания). Регистрировали фоновую запись ЭЭГ в альфа- и тета-диапазонах. Статистическую обработку проводили методами непараметрического сравнения по критерию Манна–Уитни. Получены результаты, свидетельствующие о достоверных отличиях группы курсантов-иностранцев от контроля по спектральным показателям альфа- и тета-ритмов. Программа закаливания была эффективна в группе курсантов-россиян, что отразилось на стабильности церебрального гомеостаза при некоторой степени неустойчивости нейродинамических процессов в ЦНС. У курсантов-иностранцев систематические холодовые тренировки привели к выраженной дезинтеграции корково-подкорковых и внутрикортикальных взаимоотношений, а также формированию двухъядерной альфа-тета-структуры фоновой ЭЭГ.

Ключевые слова: ЭЭГ, альфа-ритм, тета-ритм, адаптация, холодовая устойчивость организма, курсанты

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Estimation of the effects of cold environment on body's functional state in humans and assessment of the body's regulatory system response to cold are the important components of the integrated picture of the stress effects of high-latitude climate on human health, well-being and performance.

The phenomenon of cold exposure training and understanding of physiological mechanisms underlying the human body transition from the not adapted to cold state to the state of stable cold resistance are the important elements of cold stress research. In this regard, it is important to consider the cost of adaptation (allostatic load) paid by humans when adjusting to the acute or prolonged exposure to low temperatures. The studies of thermal homeostasis in both Russian students (young men, who were born and permanently reside in the climate of West Siberia) and foreign students coming from the regions with tropical and subtropical climate are of great interest [1, 2]. Today, geographical migration of the youth from the countries of Africa, Asia, Middle East aimed at getting primary

education in the RF shows an upward trend [3]. However, there is almost no description of the types of adaptation (acclimation, acclimatization) to sudden temperature changes associated with the change in geographical conditions. At the same time, there is little research on the body's functional states and the dynamics of morbidity among foreigners, especially those, who attend military higher education institutions, relative to students living in the RF.

Bioelectric activity of the brain recorded by EEG is a sensitive and, which is more important, informative indicator of body's adaptive changes and compensatory processes in the central nervous system (CNS) [4–8]. The effects of low temperatures on formation of the brain bioelectric activity, specific EEG patterns in individuals constantly or from time to time exposed to cold are reported in a number of domestic [9–11] and foreign studies [10–12]. Thus, the increase in total spectral power of alpha and theta waves was observed in the EEG patterns of one group of subjects under exposure to cold [7]. The other study, in contrast, reported the cold related decrease in the alpha rhythm power [10]. The literature provides conflicting data on the impact of hypothermia and regular cold exposure training on bioelectric activity of the brain, which are largely dependent on the aspect of the issue considered (acute hypothermia or prolonged exposure, deliberate cold exposure training or inevitable impact of cold high-latitude climate, study of aboriginal or immigrant community, etc.). Furthermore, the issue of the search for EEG markers of successful adjustment of humans to cold exposure is still relevant.

The study was aimed to assess the impact of the military academy cadets' incremental exposure to the graduated cold stress (cold exposure training) on the EEG spectral characteristics in the alpha and theta ranges.

METHODS

The cross-sectional study involved 58 subjects divided into three groups. The first group of 18 individuals (hereinafter, control group, civil students, CG) consisted of young men studying at the University of Tyumen, who were permanent residents of the Tyumen region; their average age was 21.36 ± 1.83 years. The second group of 29 individuals (hereinafter, experimental group 1 undergoing cold exposure training, military cadets from various regions of the RF, CETG1) consisted of military cadets studying at the Proshlyakov Tyumen Higher Military Engineer Command School, who were permanent residents of the Tyumen region and the regions of the Urals or Siberian Federal District of the RF; their average age was 20.58 ± 1.29 years. The third group of 11 individuals (hereinafter, experimental group 2 undergoing cold exposure training, foreign military cadets, CETG2) consisted of military cadets studying at the Proshlyakov Tyumen Higher Military Engineer Command School, who were permanent residents of the countries with tropical and subtropical climate (Nicaragua, Congo, Mozambique, Guinea, Guinea-Bissau, Gabon) and had dark skin; their average age was 23.39 ± 1.95 years.

Inclusion criteria: health status group 1–2; good academic performance (learning success as a criterion of primary activity efficiency). Exclusion criteria: history of neurological disorder and/or exacerbation of any disease within two weeks before the study.

The study was conducted in mid-November. It was preceded by the 2.5 month training aimed to improve cold resistance in military cadets of both experimental groups in accordance with the program of incremental exposure to the graduated cold stress aimed at improving the servicemen's adaptive capacity. The program included the entry level training aimed to improve cold resistance (regular dousing with cold (+5…+8 °C) water outdoors, training was conducted 2–3 times a week throughout 3 weeks) [13] and the special training (immersion in cold (+2…+2.5 °C) water in full combat gear, training was conducted 1–2 times a week throughout 2 months) [14].

The stationary Neuron-Spectrum-4/EPM EEG system (Neurosoft; Russia) was used for EEG recording. A total of 16 active electrodes were placed over two hemispheres following the 10–20 system, unipolar configuration with ear references was used. Background EEG was recorded in the relaxed wakefulness states with the eyes closed in the soundproof darkened room. The recording range was 0.5–35 Hz. The electrode resistance was < 20 kOhm. The sampling rate of 500 points was used. The background EEG recording was assessed based on the 20 epochs, 10–15 s each, by selecting the artifact-free segments. The EEG mathematical analysis was performed using the Neuron-Spectrum software for Fourier transform in the θ (4.0–8.0 Hz) and α (8.0–14.0 Hz) frequency ranges. The total spectral power (μV2), rhythm indices (Hz), integrated EEG alpha/theta indices were used to describe the functional state of the subject's brain.

Statistical data processing was performed using the SPSS Statistics 23 software package. The data were presented as median (Me), first and third quartiles (Q1–Q3). The distribution was tested for normality using the Shapiro–Wilk test. The distribution of indicators was non-normal, therefore, comparison with controls was performed by nonparametric methods using the Mann–Whitney U test for two independent groups.

RESULTS

Visual assessment of the subjects' EEG recordings allowed us to distinguish the major variants of the brain bioelectric activity organization in accordance with the classification by E.A. Zhirmunskaya [15] (Fig. 1).

In the control group, type I bioelectric activity (66% of cases) showing the organized alpha activity structure and the pronounced anterior-posterior gradient of the background rhythm prevailed. Type II and III EEG patterns were less frequent: 28 and 6% of cases, respectively. In the experimental groups 1 and 2, which were through cold exposure training, a hypersynchronous EEG type showing the evident alpha rhythm with no spindle events was the most common (55 and 64%, respectively). In 20% of cases, young men in the experimental group 1, who underwent cold exposure training, also showed a type III asynchronous EEG pattern with the sharp decrease in alpha rhythm and substitution of alpha rhythm with oscillations in the theta and beta ranges.

The EEG spectral power analysis in the theta range revealed significantly higher values in the right hemispheric leads in the experimental group 2 undergoing cold exposure training compared to controls (Fig. 2).

Thus, the subjects in CETG2 were significantly superior to the CG in theta activity recorded by the following electrodes: right anterior frontal Fp_2 ($U = 26$; $Z = –3.28$; $p < 0.001$), right frontal F4 ($U = 29$; $Z = –3.14$; $p = 0.002$), right central C4 ($U = 31$; $Z = –3.05$; $p = 0.002$), right anterior temporal F_8 ($U = 49$; $Z = –2.24$; $p = 0.024$), right temporal T_4 ($U = 10$; $Z = –4.00$; $p < 0.001$), right parietal P_4 ($U = 24$; $Z = –3.37$; $p = 0.001$), and right occipital O_2 ($U = 45$; $Z = –2.42$; $p = 0.015$). Young men in CETG1, on the contrary, had significantly lower total spectral power values in theta range compared to controls based on the activity recorded by the following electrodes: left and right

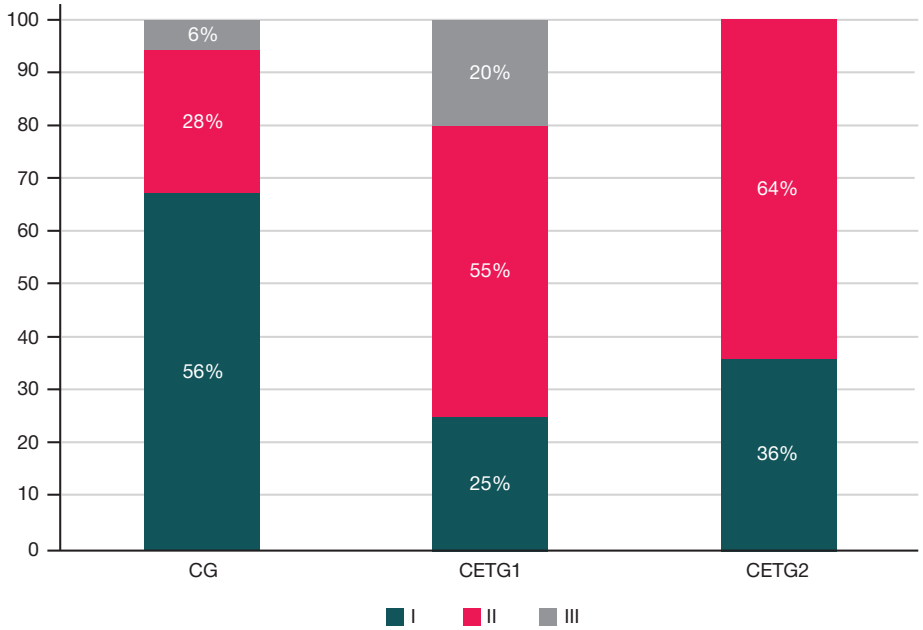

Fig. 1. Proportions of various EEG types in the samples of subjects [1]. CG — control group, CETG1 — experimental group 1 undergoing cold exposure training, CETG2 — experimental group 2 undergoing cold exposure training

anterior frontal Fp_1 (U = 119.5; Z = –3.09; p = 0.002), Fp_2 (U = 120; Z = –3.07; p = 0.002), left central C_3 (U = 165; Z = –2.10; p = 0.035), right anterior temporal F_8 (U = 161; Z = –2.17; p = 0.029), left and right temporal T_3 (U = 142; Z = –2.60; p = 0.009), T4 (U = 151; Z = –2.39; p = 0.016), left and right posterior temporal T_5 (U = 112; Z = –3.26; p = 0.001), T_6 (U = 173; Z = –1.92; p = 0.050).

In the alpha band, the total spectral power was also higher in CETG2, it was significantly superior to that of CG in the following leads: right anterior frontal Fp_2 (U = 23; Z = –3.41; p < 0.001), right frontal F_4 (U = 24; Z = –3.37; p < 0.001), right central C_4 (U = 46; Z = –2.38; p = 0.017), right anterior temporal F_8 (U = 33; Z = –2.96; p = 0.003), left and right temporal T_3 (U = 54; Z = –1.95; p = 0.050), T_4 (U = 13; Z = –3.86; p < 0.001) (Fig. 3).

The total spectral power in the alpha range of the CETG1 subjects was significantly lower compared to that of controls based on the activity recorded by the following electrodes: left temporal T_3 (U = 170; Z = –1.98; p = 0.042) and left posterior temporal T_5 (U = 171; Z = –1.92; p = 0.050).

The theta index of the experimental group 2 undergoing cold exposure training exceeded the values of the control group over the almost entire convex surface, except for C3, F8 and T5, based on the activity recorded by the following electrodes: left and right anterior frontal Fp_1 (U = 28; Z = –3.19; p = 0.001), Fp_2 (U = 7; Z = –4,13; p < 0,001), left and right frontal F_3 (U = 53; Z = –2.06; p = 0.038), F_4 (U = 44; Z = –2.47; p = 0.013), right central C_4 (U = 20; Z = –3.55; p < 0.001), right anterior temporal F_8 (U = 39.5; Z = –2.67; p = 0.007), left and right temporal T_3 (U = 42; Z = –2.56; p = 0.010), T_4 (U = 8; Z = –4.09; p > 0.001), right posterior temporal T_6 (U = 26.5; Z = –3.25; p = 0.001), left and right parietal P_3 (U = 3; Z = –4.31; p < 0.001), P_4 (U = 0; Z = –4.44; p < 0.001), left and right occipital O1 (U = 45; Z = –2.42; p = 0.015), O_2 (U = 0; Z = –4.44; p < 0.001) (Table 1).

The extent of theta oscillations in the experimental group 1 undergoing cold exposure training was the same as in the control group.

The EEG alpha index of the CETG2 subjects exceeded that of controls in frontal regions of the brain based on the activity recorded by the following electrodes: left and right anterior frontal Fp_1 (U = 50; Z = –2.17; p = 0.029), Fp_2 (U = 35; Z = –2.87; p = 0.004), right anterior temporal F_8 (U = 41; Z = –2.58; p = 0.009). Furthermore, the alpha rhythm index of the CETG2 subjects was significantly lower than that of controls in the midline and caudal regions based on the signals in the following leads: left central C_3 (U = 51.5; Z = 2.13; p = 0.033), left and right parietal P_3 (U = 47; Z = 2.33; p = 0.019), P_4 (U = 13; Z = 3.86; p < 0.001), and right occipital O_2 (U = 45; Z = 2.43; p = 0.015). In general, the alpha index of the control group 2 undergoing cold exposure training showed no frontal–occipital gradient.

The alpha index of the experimental group 1 undergoing cold exposure training had the zonal specifics and increased in the direction from frontal to caudal regions, the same as in the control group. In the CETG1 subjects, the alpha rhythm index values exceeded that of controls only in the left anterior frontal Fp_1 (U = 166; Z = 2.07; p = 0.037) and the right frontal F8 (U = 162; Z = 2.02; p = 0.042) leads.

Then we calculated the index of the cortical-subcortical neurodynamic processes' stability as an integrated ratio of alpha/theta rhythms based on the total spectral power and rhythm index (Table 2).

The highest values of the CNS functional state stability were obtained in the experimental group 1 undergoing cold exposure training, while the lowest values that were significantly different from that of the control group were reported in young men in the experimental group 2, who were through cold exposure training.

DISCUSSION

Despite certain conventionality of the classification reported in the number of studies, visual assessment of EEG makes it possible to obtain a comprehensive picture of the formation of the subjects' brain bioelectrical activity and the brain functional state [16, 17].

Our findings showed that type I EEG with the evident background brain rhythm, it's clear zonal gradation by index and power modulated into spindles prevailed in the control group consisting of civil young men. It can be assumed that the optimal functional

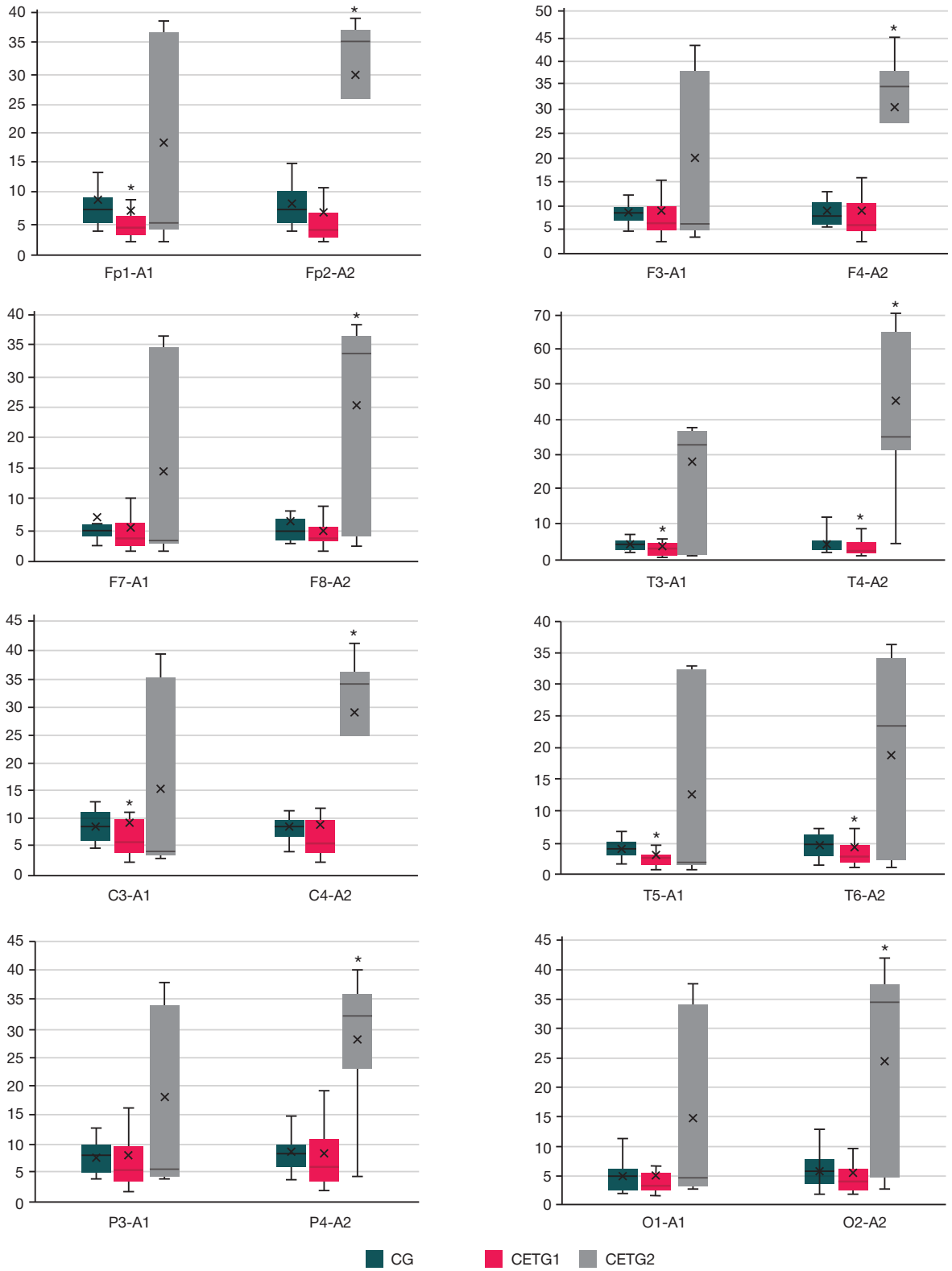

Fig. 2. Total spectral power (μV2) in the theta range (4–8 Hz) in 16 major leads. The box margins correspond to quartiles Q25% and Q75%. Lines inside the boxes — medians, crosses in the box — average values, upper and lower deviations — maximum and minimum values. * — significance of differences from the control group ($p < 0.05$) based on the Mann–Whitney U test. CG — control group, CETG1 — experimental group 1 undergoing cold exposure training, CETG2 — experimental group 2 undergoing cold exposure training

state of the CNS associated with the balanced interaction between the activating mesencephalic and synchronizing diencephalic brain structures was observed in the majority of the control group members. The type II hypersynchronous (monorhythmic) EEG was more often found in two experimental groups of military cadets, which suggested some strain of adjustment mechanisms, rhythmic disorganization, and redistribution of the limbic-diencephalic, thalamocortical and intracortical interactions in the subjects' brain. The increase in the share of individuals with monorhythmic EEG in our experimental samples and the fact of detecting type III asynchronous (flat) EEG pattern in CETG1 could result from the cadets' (both Russian and foreign) body incomplete adaptation

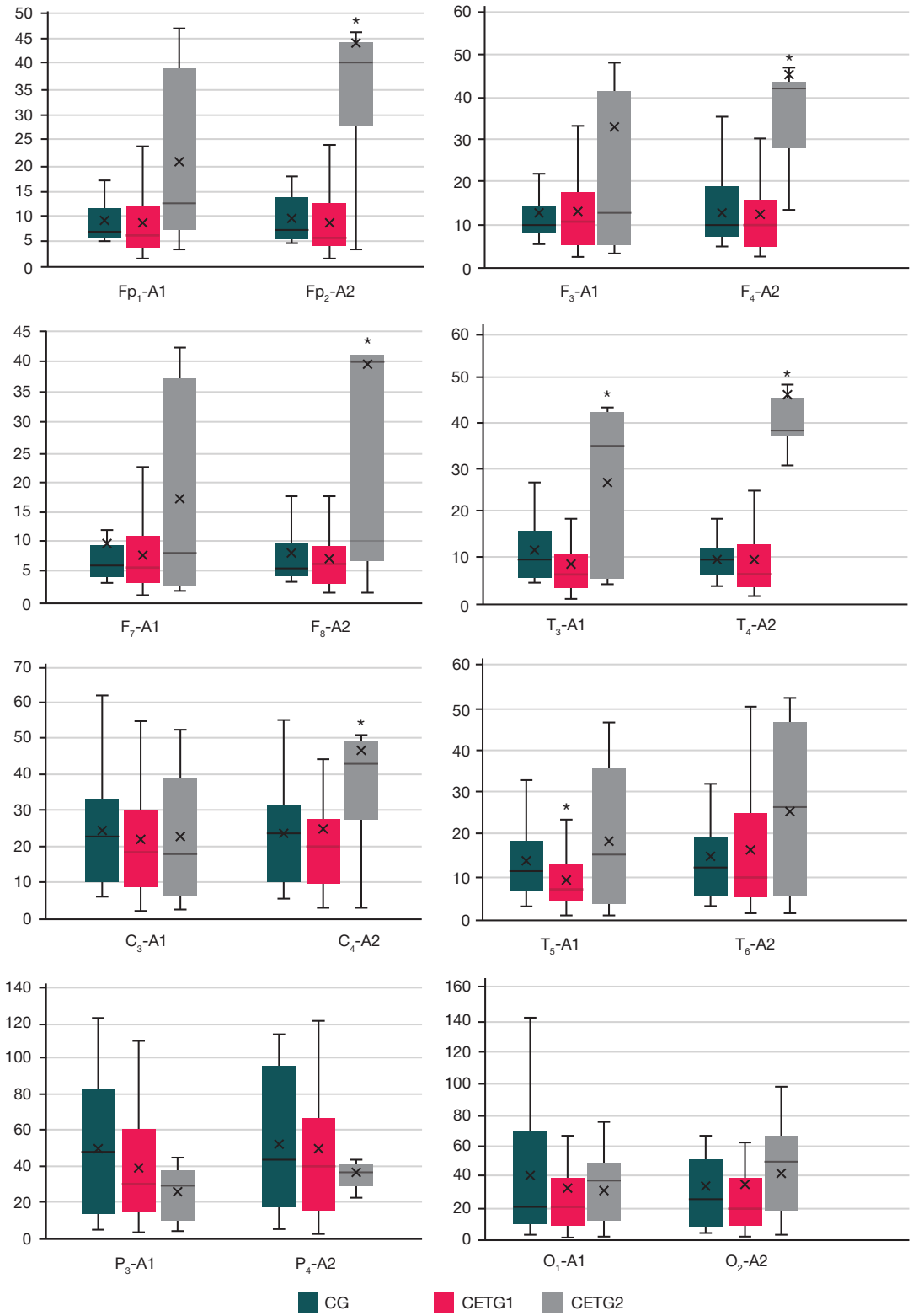

Fig. 3. Total spectral power (μV2) in the alpha range (8–14 Hz) in 16 major leads. The box margins correspond to quartiles Q25% and Q75%. Lines inside the boxes — medians, crosses in the box — average values, upper and lower deviations — maximum and minimum values. * — significance of differences from the control group ($p < 0.05$) based on the Mann–Whitney U test. CG — control group, CETG1 — experimental group 1 undergoing cold exposure training, CETG2 — experimental group 2 undergoing cold exposure training

to the regular cold exposure training conducted as part of the special training program.

The researchers have noted that the CNS plays a key role in the process of cold adaptation and cold exposure training, which is reflected in the brain biorhythm pattern [1]. The other paper also reports that specific EEG patterns that are considered as adaptive can develop in humans due to prolonged cold exposure (as well as due to acute cold exposure) [7].

A number of studies demonstrate high sensitivity of the EEG spectral power in theta and alpha ranges to the stress exposures experienced by the body, which are essentially the markers of successful adaptive responses to various adverse environmental factors [18–20].

Table 1. EEG theta and alpha rhythm indices (%) in major leads. Median (quartiles Q25%; Q75%)

Electrode	Rhythm index, %	CG, $n = 18$	CETG1, $n = 29$	CETG2, $n = 11$
Fp_1	θ (4.0–8.0 Hz)	11.35 (7.95; 12.50)	12.70 (10.40; 17.80)	18.60 (14.95; 23.65)*
	α (8.0–14.0 Hz)	10.10 (7.17; 18.52)	17.73 (11.20; 24.00)*	25.80 (14.95; 33.33)*
Fp_2	θ (4.0–8.0 Hz)	9.80 (7.20; 13.57)	11.30 (9.10; 16.90)	23.10 (21.80; 23.80)*
	α (8.0–14.0 Hz)	8.90 (7.62; 18.65)	17.60 (9.90; 26.00)	27.20 (25.15; 27.50)*
F_3	θ (4.0–8.0 Hz)	16.05 (15.12; 20.67)	19.10 (14.80; 23.50)	21.10 (16.40; 25.05)*
	α (8.0–14.0 Hz)	23.50 (17.72; 28.70)	26.40 (16.00; 35.10)	26.50 (21.05; 35.05)
F_4	θ (4.0–8.0 Hz)	15.10 (13.10; 21.17)	17.60 (15.40; 22.80)	23.20 (22.60; 23.50)*
	α (8.0–14.0 Hz)	22.95 (18.37; 26.70)	28.00 (16.80; 35.50)	26.70 (24.75; 29.05)
F_7	θ (4.0–8.0 Hz)	15.05 (12.30; 17.97)	15.40 (12.20; 20.20)	17.90 (14.55; 23.45)
	α (8.0–14.0 Hz)	19.85 (14.65; 27.65)	22.90 (14.50; 28.30)	26.60 (15.90; 31.95)
F_8	θ (4.0–8.0 Hz)	12.20 (9.27; 17.77)	15.00 (9.90; 20.60)	23.70 (18.95; 24.20)*
	α (8.0–14.0 Hz)	16.45 (10.57; 22.72)	21.60 (17.40; 31.80)	27.90 (26.05; 28.90)*
T_3	θ (4.0–8.0 Hz)	13.90 (12.12; 15.92)	14.60 (11.11; 17.90)	24.20 (13.95; 25.00)*
	α (8.0–14.0 Hz)	30.75 (22.97; 43.37)	30.20 (20.00; 38.70)	28.20 (27.50; 30.80)
T_4	θ (4.0–8.0 Hz)	12.85 (10.27; 16.72)	13.40 (10.10;16.80)	23.70 (23.45; 29.50)*
	α (8.0–14.0 Hz)	26.35 (20.97; 37.45)	28.90 (18.90; 43.00)	26.80 (18.85; 27.55)
C_3	θ (4.0–8.0 Hz)	14.80 (13.15; 17.45)	15.30 (11.60; 18.10)	18.30 (13.55; 21.35)
	α (8.0–14.0 Hz)	37.40 (26.22; 48.75)	45.00 (31.10; 47.80)	27.20 (14.50; 33.45)*
C_4	θ (4.0–8.0 Hz)	14.45 (12.12; 16.67)	13.60 (10.80; 17.70)	23.00 (22.25; 24.20)*
	α (8.0–14.0 Hz)	37.95 (23.92; 50.72)	40.90 (31.30; 50.20)	29.00 (26.90; 31.60)
T_5	θ (4.0–8.0 Hz)	11.95 (10.32; 14.90)	11.80 (9.60; 15.60)	13.00 (11.00; 20.25)
	α (8.0–14.0 Hz)	39.70 (29.45; 48.55)	37.60 (25.60; 45.20)	29.20 (22.25; 37.00)
T_6	θ (4.0–8.0 Hz)	10.90 (8.95; 12.90)	11.10 (8.30; 17.10)	21.00 (14.45; 23.85)*
	α (8.0–14.0 Hz)	35.25 (22.92; 50.65)	41.30 (27.50; 54.30)	30.20 (22.55; 31.45)
P_3	θ (4.0–8.0 Hz)	8.65 (7.57; 12.00)	10.10 (7.90; 13.20)	23.40 (21.50; 27.00)*
	α (8.0–14.0 Hz)	55.90 (30.62; 67.15)	54.30 (41.40; 64.80)	29.00 (18.40; 35.95)*
P_4	θ (4.0–8.0 Hz)	9.60 (8.12; 11.47)	10.80 (7.50; 13.70)	33.50 (23.85; 34.75)*
	α (8.0–14.0 Hz)	54.95 (42.15; 63.05)	49.30 (40.50; 68.10)	21.30 (18.35; 27.35)*
O_1	θ (4.0–8.0 Hz)	7.45 (6.45; 10.72)	9.30 (7.00; 12.90)	16.10 (10.95; 18.80)*
	α (8.0–14.0 Hz)	57.30 (41.47; 66.77)	53.90 (40.50; 66.80)	30.10 (24.45; 45.85)
O_2	θ (4.0–8.0 Hz)	9.15 (7.37; 11.22)	9.90 (6.70; 13.50)	19.30 (16.60; 22.75)*
	α (8.0–14.0 Hz)	48.45 (32.57; 63.42)	45.10 (38.70; 64.60)	31.80 (24.20; 38.55)*

Note: * — significance of differences from the control group ($p < 0.05$) based on the Mann–Whitney U test. CG — control group, CETG1 — experimental group 1 undergoing cold exposure training, CETG2 — experimental group 2 undergoing cold exposure training.

In our study, foreign military cadets demonstrated significantly higher total spectral power in the theta and alpha bands in the right hemisphere compared to controls, which could be indicative of the increased autonomic nervous system sympathetic division activation. It is well known that the right hemisphere modulates the sympathetic tone [7], while the autonomic nervous system integrates the functions of internal organs via activation of autonomic centers in the brain [21]. At the same time, the increase in the theta rhythm total power reflects the decrease in the cortical centers' inhibitory control over the brainstem and subcortical structures [22], and communication between the autonomic nervous system and the overlying nervous centers plays a vital role in developing an adaptive response to cold exposure during the cold exposure training [23].

The alpha rhythm abundance (index) and frontal asymmetry represent an important indicator of stress exposure [24]. In our study, alpha rhythm showed significant asymmetry of the

Table 2. Integrated EEG indices (alpha/theta) averaged over all leads. Median (quartiles Q25%; Q75%)

Groups of subjects	Total spectral power (μV^2)	Rhythm index (%)
CG	1.85 (1.41; 2.63)	2.71 (1.88; 3.23)
CETG1	2.41 (1.19; 3.14)	2.56 (1.38; 3.87)
CETG2	1.14 (1.05; 1.73) $p < 0.047$	1.13 (1.07; 1.55) $p < 0.001$

Note: p — significance of differences from the control group based on the Mann–Whitney U test. CG — control group, CETG1 — experimental group 1 undergoing cold exposure training, CETG2 — experimental group 2 undergoing cold exposure training.

total spectral power and rhythm index values in the right frontal and anterior temporal leads in the group of foreign military cadets (CETG2). It is believed that the emergence of the alpha oscillation frontal asymmetry pattern with increasing bioelectric activity of the right hemisphere is associated with the avoidance system responses and the aggressive-defensive behavioral stereotypes, while alpha activation of the left hemisphere reflects the approach system activity and the orientative-exploratory behavior [25].

There are some research and theoretical papers that consider not the EEG spectral characteristics themselves, but their relationships defined as rhythm indices, as biomarkers of stress [26, 27]. Interaction of the brain bioelectric activity main spectral components can be used as a marker of the homeostatic and adaptation mechanisms' efficiency [28, 29]. One of such indices, characterizing the adaptation processes and maladaptive disorders, represents the ratio of the alpha/theta oscillation spectral characteristics.

In our groups of subjects, a functional "α-core" was formed in the samples of civil students and Russian military cadets based in the alpha/theta index, while foreign military cadets showed signs of the α-core disruption by other EEG spectral components, specifically the theta range. Such pattern could be indicative of destabilization of the neurodynamic processes in the CNS associated with the cerebral homeostasis disturbance and incomplete adaptive adjustment of the foreign cadets' bodies under the influence of the extreme cold weather training programs. Subdominance of alpha and theta components in CETG2 resulted in the formation of binary α-θ structure of brain biorhythms typical for maladaptation with the following further scenarios: transition to the adjusted and cold resistant (weathered) state and formation of α-core on the EEG or transition from early-stage maladaptation to persistent disorder [30].

CONCLUSIONS

The combination of EEG spectral characteristics in alpha and theta ranges, as well as their interaction during formation of the brain background bioelectric activity α-core suggest that the special program involving incremental exposure of military academy cadets to the graduated thermal stress ensured the desired weathering effect in young Russians, which affected the cerebral homeostasis stability with some instability of neurodynamic processes in the CNS. In foreign military cadets, regular cold exposure training resulted in the pronounced disintegration of cortical-subcortical and intracortical interactions, as well as in the formation of binary α-θ structure of background EEG, suggesting incomplete adaptation processes and high stress load on the body. We have prepared some recommendations based on the findings. First, it is reasonable to extend the entry level cold resistance training of foreign military cadets from 3 weeks to 3–6 months in order to ensure milder adaptation to the graduated short-term cold stress (extend the acclimation period). Second, the findings should be compared with the EEG data of foreign military cadets not engaged in cold exposure training, who are in the same situation of adaptation to the climatic and geographical conditions of West Siberia, in further studies.

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CORRELATIONS BETWEEN SERUM LEVELS OF HISTAMINE, DIAMINE OXIDASE, SUBSTANCE P IN PATIENTS WITH CHRONIC URTICARIA

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The onset and progression of various disorders, including chronic urticaria, are associated with stress. The gut-brain-skin axis is used to describe correlations among the nervous system, gastrointestinal tract states and systemic and skin inflammation. We have summarized inflammatory and immune mechanisms underlying chronic urticaria and stress in the context of the gut-brain-skin axis. The study was aimed to show the relationships between substance P, the neurotransmitter, and diamine oxidase, the enzyme disrupting histamine in the gut of patients suffering from chronic urticaria. A total of 165 adults aged 18–68 were enrolled; 97 patients had chronic urticaria, the comparison group was formed of 68 nominally healthy individuals. ELISA (Cloud-Clone Corp; China) was used to simultaneously estimate serum levels of substance P, diamine oxidase, and histamine. We revealed a significant positive correlation ($\rho = 0.5$; $p < 0.05$) between substance P and diamine oxidase in patients with chronic urticaria and in the comparison group, which confirmed the existence of the gut-brain-skin axis. The paper provides theoretical background and new targets for treatment of chronic urticaria. The possibility of prevention and treatment of these disorders by modulation of gut microbiota is discussed, the place of diet and the lifestyle modification contributing to improvement of general health are determined.

Keywords: substance P, diamine oxidase, histamine, chronic urticaria, stress, gut-brain-skin axis

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Compliance with ethical standards: the subjects submitted the informed consent to participation in the study approved by the Ethics Committee of the Nikiforov's All-Russian Center for Emergency and Radiation Medicine, EMERCOM of Russia (protocol № 6/21 dated 24 June 2021).

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ВЗАИМОСВЯЗЬ УРОВНЕЙ ГИСТАМИНА, ДИАМИНОКСИДАЗЫ И СУБСТАНЦИИ Р В СЫВОРОТКЕ КРОВИ У ПАЦИЕНТОВ С ХРОНИЧЕСКОЙ КРАПИВНИЦЕЙ

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Возникновение и прогрессирование различных заболеваний, в том числе хронической крапивницы, связаны со стрессом. Ось кишечник-мозг-кожа используют для объяснения корреляций между состоянием нервной системы, желудочно-кишечного тракта, а также системным и местным воспалением в коже. В контексте оси кишечник-мозг-кожа мы обобщили воспалительные и иммунные механизмы хронической крапивницы и стресса. Целью нашего исследования было показать взаимосвязь между нейротрансмиттером субстанцией Р и диаминоксидазой, ферментом, разрушающим гистамин в кишечнике у пациентов, страдающих хронической крапивницей. В исследование было включено 165 взрослых людей от 18 до 68 лет, 97 пациентов страдали хронической крапивницей, группу сравнения составили 68 условно здоровых лиц. Методом ИФА (Cloud-Clone Corp; Китай) одновременно оценивали уровни субстанции Р, диаминоксидазы и гистамина в сыворотке крови. Была выявлена прямая заметная корреляционная связь ($\rho = 0,5$; $p < 0,05$) между субстанцией Р и диаминоксидазой у пациентов, страдающих хронической крапивницей, и в группе сравнения, что подтвердило наличие оси кишечник-мозг-кожа. В статье представлены теоретическая основа и новые цели для лечения хронической крапивницы. Обсуждена возможность предотвратить и лечить эти патологические состояния путем модуляции микробиоты кишечника, определены место диеты и изменения образа жизни, способствующие улучшению состояния здоровья в целом.

Ключевые слова: субстанция Р, диаминоксидаза, гистамин, хроническая крапивница, стресс, ось кишечник-мозг-кожа

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Соблюдение этических стандартов: от испытуемых было получено информированное согласие на участие в исследовании, которое было одобрено Комитетом по этике ФГБУ ВЦЭРМ имени А. М. Никифорова МЧС России (протокол №6/21 от 24 июня 2021 г.).

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The fast-paced life of today's society and recent events starting from the pandemic of novel coronavirus infection cause severe anxiety and stress. This results in numerous adaptive physiological alterations of the cardiovascular, endocrine, nervous systems, thereby significantly disturbing the human body's allostasis. The essence of allostasis is that physiological systems continuously fluctuate to adjust to the environment [1]. Physical and psychological stress can be acute or chronic,

depending on the duration and intensity. Acute stress in associated with a sudden, short-term, isolated, unique incident, such as a traffic accident, surgical intervention [2]. Chronic stress results from the long-term and frequently repeated exposure to psychogenic or physiological stressors. This causes endocrine and behavioral responses regulated by various neurochemical systems. Strong association between stressful life events and disorders of the cardiovascular, endocrine, nervous, respiratory systems, cancer, gastrointestinal tract and skin disorders is well known [3].

The term "axis" was accepted as the one describing a two-way relationship between the nervous system and other systems, such as gastrointestinal tract, skin. The gut-brain axis is the best-studied one. The concept of the gut-brain axis includes not only classical autonomic nervous system pathways, sympathetic and parasympathetic, but also endocrine interactions (hypothalamic-pituitary-adrenal axis), connections between cognitive and emotional functions in the brain [4]. Communication involves the enteric nervous system, metabolic pathways [5]. Mast cells are important effector cells of the gut-brain axis. When exposed to stress, these cells release a broad range of neurotransmitters and pro-inflammatory cytokines capable of affecting the gastrointestinal tract physiology [6]. Activation of the vagus nerve by cytokines stimulates anti-inflammatory responses of neurons, since acetylcholine, the main neurotransmitter of the vagus nerve, impairs the release of such cytokines, as tumor necrosis factor alpha (TNFα), interleukin 1β (IL1β), IL6, and IL18. The immune cells produce various neurotransmitters, thereby affecting serotonergic systems, regulate mood and behavior. For example, leukocytes synthesize and release corticotropin and endorphins in response to bacterial lipopolysaccharides [4]. Stress and sadness modulate hunger and dietary habits. High calorie foods can improve the well-being. Food also represents an important factor affecting the gut microbiome [7].

Microbiome plays an important role in human health, homeostasis, immune system, and disease pathogenesis [7]. The disrupted link between microbiome and the host body has been actively studied in individuals with gastrointestinal tract disorders. The researchers have shown that chronic stress activates caspase-1, thereby affecting the gut microbiome composition, which results in the reduced abundance of *Akkermansia spp*. and *Blautia spp*. and the increase in the ratio of *Firmicutes/Bacteroidetes* [8], *Escherichia coli*, and *Bacteroides fragilis* [9]. The type I IL1 receptor and its ligands are expressed in the brain regions responsible for brain response to stress and transmission of IL1β signals, which is fundamental for mediating neurobehavioral and neuroendocrine responses to stress and adaptation. Various stressors activate inflammation via NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) or P2X7 (purinergic ligand-gated ion channel 7 receptor) receptors, which results in maturation of caspase-1 causing the IL1β and IL18 release. The levels of caspase-1 and NLRP3 mRNA are elevated in blood cells of patients with depression [8]. Depression and anxiety are the best understood mental disorders associated with the gut-brain axis. It has been confirmed that the tryptophan precursor suppression is associated with the gut microbiome. Considering the antimicrobial and anti-inflammatory effects associated with the gut microbiota restoration using antidepressants, it was proposed to treat depression and anxiety disorders through manipulation of microbiome and the gut-brain axis [4]. In the recent study, the relationship between the gut microbiome disruption severity and the severity of cognitive impairment was assessed in children with autism spectrum disorder [10]. The other study showed that gut microbiota composition alteration could contribute to the development of neurodegenerative process [11].

Diet plays an important role in determining the gut microbiota composition. Metabolites produced by gut microbiota not only modulate the mucosal immune response, but also affect lungs and the brain. Microaspiration of bacteria or travel of the sensitized immune cells through lymph and blood can also affect the immune response of other organs. Gut dysbiosis is associated with a number of lung diseases, including asthma and cystic fibrosis. The two-way relationships between the gut and lungs (gut-lung axis) are exemplified by the intestinal issues observed in individuals with lung diseases. The study has shown that mucosal immune cells can migrate through the lymphatic system, which determines the immune response of various organs (gastrointestinal tract, lungs, etc.). The T and B cells of Peyer's patches can travel through bloodstream and migrate to both intestine and extraintestinal sites (including bronchial epithelium and lymphoid tissues) [12]. It has been shown that immune cells (T and B cells that secrete sIgA and ensure mucosal immunity) found in the lamina propria of the gut and mesenteric lymph nodes neutralize the majority of translocating bacteria, however, fragments of dead bacteria travel from the mesenteric lymphatic system to systemic circulation. These bacterial fragments and metabolites can modulate immune response in the lung [13]. Perhaps, there is a similar pathophysiological pattern involving other systems of human body.

There is emerging research on the skin microbiome and its association with the gut [14]. There are papers describing the gut-brain-skin axis [15]. Thus, the correlations among gut mictobiota, emotional states and systemic and skin inflammation have been reported. Gut dysbiosis contributed to the Th17-mediated skin inflammation via the IL23, IL17 signaling pathway by increasing production of IL22 and interferon gamma (IFNγ), which resulted in hyperproliferation of keratinocytes [16]. The skin responses caused by stress primarily involve cytokine (for example, IL6, IL1, IFNγ) secretion and activation of peripheral corticotrophin-releasing hormone of the skin produced by sebocytes, keratinocytes, and mast cells [17]. The nervous, endocrine, and immune systems have many common mediators (for example, neurotransmitters, neuropeptides, hormones, cytokines) capable of modulating the nervous system activity during stress [18, 19]. Peripheral nerves in the skin mediate neurogenic inflammation by releasing neuropeptides (substance P (SP), brain-derived neurotrophic factor, and nerve growth factor). Thus, SP is a pro-inflammatory neuropeptide that is related to stress. The SP biological activity is mediated primarily by the neurokinin receptors (NK)-1. The SP/NK-1 receptor pathway can be activated in response to the stress stimulation of both autonomic and central nervous systems. The study has shown that NK-1 is expressed mostly in mast cells, which suggests the important role of the NK-1 receptor activation in the mast cell degranulation caused by stress [20]. Other researchers have shown that SP can be involved in the corticotrophin-releasing hormone-mediated mast cell degranulation during stress [21]. It has been also shown that SP can increase virulence of the skin microbiome due to alterations in bacterial cytoskeleton, which can represent one more mechanism contributing to its role in neurogenic inflammation [22]. High SP levels were observed in depression suggesting that the depression pathogenesis was associated with SP/NK-1 [20].

Great interest in the issue is indicative of its relevance, therefore, the study was aimed to show the relationship

Table. Correlations between the indicators Histamine — Diamine oxidase, Substance P — Diamine oxidase in patients included in the study on chronic urticaria

Indicator	Histamine — Diamine oxidase	Substance P — Diamine oxidase
Correlations in the comparison group (n = 68)	–0.4	0.5
Correlations in the group of patients with chronic urticaria (n = 97)	–0.4	0.5
Correlations for all people included in the study (n = 165)	–0.4	0.5

Note: ρ — degree of correlation.

between the SP neurotransmitter, and diamine oxidase, the enzyme disrupting histamine in the gut of patients suffering from chronic urticaria.

METHODS

The study involved assessment of substance P, diamine oxidase, and histamine in patients suffering from chronic urticaria. Enzyme-linked immunoassay (Cloud-Clone Corp; China) was used to simultaneously estimate three serum inducators in patients with chronic urticaria and in the comparison group. A total of 165 adults aged 18–68 were enrolled. Among them 97 patients with chronic urticaria had been receiving outpatient treatment at the Nikiforov's All-Russian Center for Emergency and Radiation Medicine, EMERCOM of Russia, in 2018–2023. Recurrent urticaria and/or angioedema occurring throughout 6 weeks or more were considered to be the inclusion criteria. The diagnosis of chronic urticaria was established in accordance with the Federal Clinical Guidelines on the Diagnosis and Treatment of Urticaria [23]. The comparison group consisted of 68 nominally healthy individuals matching those of the index group in gender and age with no signs of urticaria or allergic disorders.

Medical and social history of each subject was taken; great attention was paid to the triggers of chronic urticaria, the existing comorbidity. Food intolerance was a trigger in 46 patients suffering from chronic urticaria out of 97; 27 patients reported stress as a trigger of the disease.

The R program for Windows was used for statistical data processing, the correlations among the studied substance P, diamine oxidase and histamine were assessed using the Spearman's rank correlation coefficient. The Chaddock scale was used for more accurate assessment of correlation strength. The differences between the indicators compared were significant at p-value < 0.05.

RESULTS

We assessed correlations among the studied substance P, diamine oxidase and histamine using the Spearman's rank correlation coefficient. The correlations of diamine oxidase with histamine and diamine oxidase with substance P turned out to be significant (p < 0.05) (Table).

The correlation analysis yielded a moderate negative correlation between histamine and diamine oxidase, the enzyme ensuring histamine degradation. A significant positive correlation between substance P and diamine oxidase attracts attention (Figure).

DISCUSSION

The substance P neuropeptide can form an almost direct link between the skin and the brain. The diamine oxidase enzyme synthesized by apical cells of the intestine can be affected by various mechanisms, from genetic suppression of activity to the impact of microbiome [24]. Recently, diamine oxidase has been proposed as a marker of mucosal integrity [25].

When reviewing the literature, we have found only one paper reporting simultaneous assessment of substance P and diamine oxidase [26]. In 2023, the results of the experimental study, during which the authors studied irritable bowel syndrome (IBS) caused by stress in the rat model, were published. High expression of SP and diamine oxidase associated with IBS was reported. In recent years, it is believed that IBS is induced by a combination of factors, such as changes in visceral sensitivity, disturbances of the gastrointestinal function and the gut-brain axis, including microbiome alterations [27]. Diamine oxidase, as an enzyme produced in the villi of the intestinal mucosa, reflects disruption and repair of the epithelium, is released into blood or the intestinal lumen after the intestinal mucosa disruption and necrosis. Normal serum levels of diamine oxidase are very low, however, these increase with increasing inflammation due to the release of large amounts of the enzyme into blood. In our study we noted an upward trend in serum levels of diamine oxidase and substance P associated with exacerbation of chronic urticaria relative to the comparison group. However, we have revealed a significant increase in the levels of diamine oxidase and substance P in patients suffering from urticaria without exacerbation, which confirms persistence of inflammation during remission and requires further investigation.

CONCLUSIONS

The study has confirmed the correlations between the substance P neurotransmitter and diamine oxidase, the enzyme degrading histamine in the gut, in both patients suffering from chronic urticaria and comparison group. This confirms the existing hypothesis of the gut-brain-skin axis.

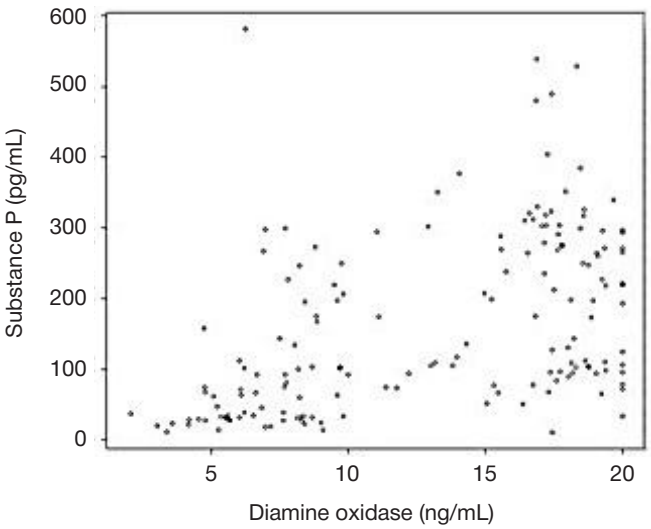

Figure. Scatter plot reflecting the correlation of substance P and diamine oxidase

However, the chronic urticaria pathogenesis represents a multifactorial complex of changes in the immune system and the gut-brain-skin axis signal transmission. The currently available approaches to treatment of urticaria are clearly defined, but not always successful. The today's guidelines on diamine oxidase deficiency are focused mainly on the diet and the use of dietary supplements. However, diet is not always effective; perhaps, in the future dietary treatment will be based on the features of human microbiome as well. The other targets of interest are represented by the effects on neurotransmitters, which will probably form the basis for treatment methods, along with the microbiome modulation following personalized profiling. It is recommended to use all the methods focused on stress correction and allostasis maintenance, including not only diet, but also adequate amount of quality sleep, positive social interactions. The effects on all the links of the gut-brain-skin axis contributing to allostasis can help recovery, since the innate adaptive plasticity of the brain can more effectively influence other systems [28].

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LASER THERAPY AND UNLOADING THERAPEUTIC GYMNASTICS IN THE TREATMENT OF DYSLIPOPROTEINEMIA

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Based on clinical practice, some patients with lipid metabolism disorders (LMD) are resistant to lipid-lowering therapy (LLT) — in such patients, taking optimal doses of LLT drugs does not reduce levels of cholesterol and its fractions to target levels and using LLT at higher doses is associated with increased odds of adverse events. To optimize the treatment, 58 patients with ischemic heart disease with LMD resistant to LLT were examined. The patients were divided into two groups: in the main group, 29 patients received laser therapy and unloading therapeutic gymnastics; in the control group, 29 patients continued to take their usual medications. The obtained results showed a significant lipid-lowering effect of the treatment in the main group: we observed a significant decrease in total cholesterol (by 27.7%, $p < 0.01$) as well as low-density lipoprotein cholesterol (by 34.7%, $p < 0.01$), a significant increase in high-density lipoprotein cholesterol (28.1%, $p < 0.01$), a significant decrease of atherogenic coefficient (by 50.2%, $p < 0.01$) and in the levels of triglycerides (by 49.6%, $p < 0.01$). At the same time, no significant positive changes in lipid profile were observed in the control group. In patients of the main group, tolerance to physical activity increased significantly, with statistically insignificant changes in the control group accordingly.

Keywords: Ischemic heart disease, lipid metabolism disorders, resistance to lipid-lowering pharmacotherapy, total triglycerides, low-level laser therapy, unloading therapeutic gymnastics, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol

Author contribution: Achilov AA — methodology development, collection and statistical processing of data, manuscript writing; Baranov AV — study concept, scientific supervision, manuscript editing; Gornov SV — study concept, scientific supervision, literature review, manuscript writing; Usmonzoda DU — data collection, literature review, manuscript writing; Achilova SA, Pulatova MD, Achilov Al.A, Mamedyarova IA — data collection, literature review; Lazechko MI — literature review, study design, manuscript editing.

Compliance with ethical standards: the study was approved by the Ethics Commitee of the Skobelkin Research and Practical Centre for Laser Medicine of the Federal Medical-Biological Agency (protocol № 2/23 dated 18 December 2023). All patients submitted the informed consent to stady participation.

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ЛАЗЕРОТЕРАПИЯ И РАЗГРУЗОЧНАЯ ЛЕЧЕБНАЯ ГИМНАСТИКА В ЛЕЧЕНИИ НАРУШЕНИЙ ЛИПИДНОГО ОБМЕНА

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В клинической практике встречаются больные с нарушениями липидного обмена (НЛО), резистентные к гиполипидемической терапии (ГТ), у которых прием оптимальных доз этих препаратов не приводит к снижению уровня холестерина и его фракций до целевого уровня, а повышение дозы препаратов способствует появлению побочных эффектов. Для оптимизации лечения таких больных было обследовано 58 больных ишемической болезнью сердца с НЛО, резистивных к ГТ. Исходно больные в зависимости от метода лечения были разделены на две сопоставимые группы: в основной 29 больных получали лазеротерапию и разгрузочную лечебную гимнастику; в контрольной — 29 больных продолжали принимать базовое медикаментозное лечение. Полученные результаты свидетельствуют о достоверном гиполипидемическом действии проведенного лечения в основной группе: отмечены достоверное снижение общего холестерина на –27,7% ($p < 0,01$) и холестерина липопротеидов низкой плотности на –34,7% ($p < 0,01$), достоверное повышение холестерина липопротеидов высокой плотности на 28,1% ($p < 0,01$), достоверное снижение коэффициента атерогенности на –50,2% ($p < 0,01$) и триглицеридов на –49,6% ($p < 0,01$). В то же время в контрольной группе достоверной положительной динамики липидограммы не наблюдали. У больных основной группы толерантность к физической нагрузке достоверно повышалась, а в контрольной группе изменялась недостоверно.

Ключевые слова: ишемическая болезнь сердца, нарушение липидного обмена, резистентность к гиполипидемической лекарственной терапии, холестерин, липопротеиды высокой плотности, липопротеиды низкой плотности, триглицериды, лазеротерапия, лечебная гимнастика

Вклад авторов: А. А. Ачилов — разработка методики, сбор и статистическая обработка материала, написание статьи; А. В. Баранов — концепция исследования, научное руководство, коррекция рукописи; С. В. Горнов — концепция исследования, научное руководство, сбор литературы, написание статьи; Д. У. Усмонзода — сбор материала и литературы, написание статьи; Ш. А. Ачилова, М. Д. Пулатова, Ал. А. Ачилов, И. А. Мамедьярова — сбор материала и литературы; М. И. Лазечко — сбор литературы, дизайн исследования и коррекция рукописи.

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Diseases of the cardiovascular system (CVS) are among the most important problems in the world. In the treatment of patients with ischemic heart disease (IHD), along with pharmacotherapy (PT), surgery methods are widely used: stenting and coronary artery bypass grafting (CABG). Using these treatment methods have a positive effect for a time and then it is gradually lost due to progressing atherosclerosis and IHD [1–3].

In recent years, significant progress has been made in PT of lipid metabolism disorders (LMD) in IHD patients. In most cases, PT has a good effect and target levels of cholesterol (CS) and its fractions are achieved [4–15]. Lipid lowering drugs (LLD) are known to be prescribed for continuous use, and this can cause side effects in the form of liver cell damage (elevated levels of transaminases: alanine aminotransferase (ALT), aspartate aminotransferase (AST)). Other side effects include myopathy, myalgia, diarrhea, nausea, liver discomfort, constipation, insomnia, headaches, etc. [16]. Dose reduction or discontinuation of LLD leads to an increase in levels of cholesterol and its atherogenic fractions. In clinical practice, some IHD patients with LMD are resistant to lipid-lowering therapy (LLT) — in such patients, taking optimal doses of the LLD does not reduce levels of cholesterol and its fractions to target values [16–19].

The above suggests that the trigger mechanisms of atherosclerosis and IHD have not yet been fully understood and require in-depth study. The existing treatment methods do not fully affect all etiopathogenetic mechanisms of atherosclerosis and IHD that contributes to the progression of the disease.

In earlier studies, it was shown that taking medications improve peripheral blood circulation at rest to normal values however, the reserve blood flow is not fully restored. Such structural changes of vessels are considered to be irreversible and serve as the most important factor in progression of cardiovascular pathology. Treatment of atherosclerosis, IHD and LMD relies on pharmacotherapy. For a more complete recovery of cardiovascular reserve and relieving the heart burden, it is advisable to use non-medication methods of treatment along with drug therapy [1–3].

It is known that laser therapy (LT) [20–24] and especially LT in combination with unloading therapeutic gymnastics (UTG) [25] can have a significant positive effect in the treatment of cardiovascular diseases (CVD). The mechanisms of such positive effect are described in many publications [20–24]. Intravenous laser blood irradiation (ILBI) at red spectrum is more often used to achieve a systemic effect while the infrared range is used for a local effect. When these two ranges are used in combination, the positive effects are cumulated.

Our observations demonstrated that the course treatment with LT did have a positive effect in the short-term period. However, four months after the course of LT, the achieved positive effect was leveled out and laboratory parameters returned to the initial values (before LT treatment course). It should be noted that antianginal therapy and LT, separately and in combination, increase blood flow and reduce peripheral vascular resistance at rest however, the reserve blood flow is not fully restored. This proves that the mentioned methods of treatment never completely recover the reserve exchange surface of capillaries. To maintain the effect of laser therapy, patients must receive a course of LT at least two or three times a year. Since such patients need lifelong treatment, they may experience certain inconveniences, difficulties or distrust and after the first or a few next courses of treatment, may not proceed with the treatment.

The main factor in restoring CV reserve is a proper selection of motor regimen for the patients. In all clinical recommendations on non-drug treatment of atherosclerosis and IHD, physical activity is of great importance. The risk of developing CVD in people leading a sedentary lifestyle is 20–50% higher than in physically active people [16]. In patients with CVD, exercise tolerance (ET) is significantly reduced due to a limited CV reserve.

The limited reserve exchange surface of capillaries can be recovered effectively with the use of UTG only. Loading exercises cause an increase in heart rate, blood pressure, respiratory rate, activation of the sympathoadrenal system undesirable for cardiac patients while unloading exercises have a normalizing effect. Severe and/or elderly patients cannot do loading exercises due to limited cardiovascular reserve and/or severity of condition but they can easily perform unloading exercises that expands the indications for the use of UTG. Such exercises are performed systematically on a daily basis, and the achieved effect lasts for a long time [1–3]. So the patients' life quality significantly improves and the number of medications taken regularly decreases accordingly — subject to our further long-term observations.

Another UTG advantage is that significant part of the heart pumping function is performed by the muscular system. The reserve capillary exchange surface area is restored and maintained due to angioneogenesis, the reserve blood flow increases, and peripheral vascular resistance is significantly reduced. Under these conditions, the heart strain is reduced [1–3]. This technique is effective even in those cases when patients develop resistance to PT. Consequently, the technique can be used for patients with coronary artery disease with LMD, in whom, despite taking LLD, it is not possible to lower blood lipids to the optimal levels due to various circumstances.

In this regard, the development and scientific substantiation of new treatment methods for common somatic diseases is one of the important and promising areas of modern medicine [26–28]. The problem is of particular importance in the case of cardiovascular pathologies, primarily atherosclerosis and coronary artery disease, especially with resistance to PT [28–32]. Using invasive treatment methods is often unjustified, and it is ineffective in the presence of severe multifocal atherosclerotic vascular lesions.

Thus, the problem of using LT and UTG in a comprehensive program of physical rehabilitation of IHD patients still contains many unresolved questions that require further investigation — the present study is an attempt to answer some of them.

The aim of the study was to develop effective ways of using red and infrared laser irradiation and UTG in rehabilitation treatment of IHD patients with LMD resistant to lipid-lowering drug therapy, with ongoing maintenance therapy.

METHODS

The study involved 58 IHD patients with stable angina pectoris II and with LMD resistant to LLT aged between 40 and 60 years (93.1% men, 6.9% women), with disease duration of 2 to 6 years. Patients with obesity, diabetes mellitus type II, arterial hypertension, chronic kidney disease and chronic renal failure were not included in the study. The distribution of IHD patients with LMD resistant to LLT by gender and age in the main group (MG) and control group (CG) is shown in Table 1.

Depending on treatment method, all patients were randomized into two groups comparable in clinical and functional characteristics and maintenance pharmacotherapy (MPT): 29 patients of the main group received a complex consisting of combined LT (ILBI and cutaneous exposure to infrared laser radiation) and kinesiotherapy in the form of UTG on MPT background; 29 patients of the control group

Table 1. Distribution of IHD patients with LMD resistant to LLD by gender and age

	Groups of patients			
	Main group (MG)		Control group (CG)	
	Number of patients	%	Number of patients	%
Men	27	93.1	27	93.1
Women	2	6.9	2	6.9
Total	29	100	29	100
Average age, years	56.5 ± 2.1		54.9 ± 2.0	

received MPT only. In both groups, if side effects occurred, LLD doses were reduced until the side effects disappeared. In each group, 12 patients (41.4%) received rosuvastatin and 17 patients (58.6%) received atorvastatin. The daily dose of statins in tablets for oral administration was 38.8 ± 2.83 mg in MG, and 39.0 ± 2.41 mg in CG.

The patients were examined using standard clinical and laboratory methods.

Before starting treatment, patients underwent laboratory examination including the detailed analysis of lipid spectrum: levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG). Atherogenic coefficient (AC) was calculated. Blood samples were drawn from peripheral elbow veins using disposable syringes. After collection, whole blood (4.5 mL) was added to a tube with 0.5 mL of 3.8% sodium citrate and mixed thoroughly. The blood was then centrifuged at 3000 rpm for 15 min. After centrifugation, plasma was collected into a clean tube and stored at –20°C. The main fractions of plasma lipids (TC, TG, HDL-C) were determined by enzymatic method using Humalyser-2000 (Human; Germany) with the following reagents from Human: cholesterol with antilipid factor (Human; Germany); triglycerides with antilipid factor (Human GmbH; Germany); HDL-cholesterol without precipitation (Human GmbH; Germany). LDL-C content was calculated using the Friedewald's formula, provided that plasma TC concentration did not exceed 4.5 mmol/L:

$$\text{LDL-C, mol/L} = (\text{TC}) - (\text{HDL-C}) - (\text{TG}/2.2)$$

according to National recommendations NSSC and SSCI (Third revision, 2007).

The results were presented in mmol/L.

Veloergometric test (VEM) was performed on a bicycle ergometer (Elema; Sweden) according to a common method of continuous stepwise increasing load according to the method developed by D. M. Aronov.

ILBI and percutaneous LT were performed using the Mustang-2000 laser therapy device (NPLC Technika LLC; Russia). We used combined LT consisting of ILBI and external infrared LT performed alternately every other day at the rate of 3 procedures per week. The total course of treatment consisted of 15 procedures (8 procedures — ILBI with 15 min exposure and 7 procedures — infrared LT with 6 min exposure). The duration of treatment was 1.5 months. For ILBI, we used a semi-conductor red laser emitting head with a wavelength of 0.63 μm, with output power of the light pipe 2 mW. For percutaneous irradiation, a semiconductor infrared laser emitting heads with a wavelength of 0.89 μm were used. The infrared radiation dose was 0.6 J/cm^2. Percutaneous LT was performed in the following zones: aorta projection — second intercostal space on the right parasternal line — 1 min; pulmonary artery projection — second intercostal space on the left parasternal line — 1 min; projection of absolute cardiac bluntness — 1 min; thoracic spine — 6 zones — 3 zones on the right and left parasternal line — 30 s each.

UTG was performed using a patented method with monitoring of blood pressure (BP), pulse and clinical condition of the patient [1, 3]. Exercises were done in portions until BP and pulse rate increased. A patient performed the first exercise by smooth bending forward in a sitting position on a chair, at a rate of up to 5 bends per minute, then had a break of 15 s, and repeated the exercise. The total number of bends on the first day was limited to 100 while each subsequent day the number of bends was increased up to 50 times bringing the total number of bends to 150–200 times per day. The patient's hands were placed on the knee joints while performing the bends. At the same time, the patient simultaneously performed flexion and extension of the arms with elements of upper limb extension. When straightened up, the patient simultaneously retracted the anterior abdominal wall and thus facilitated movement of the diaphragm, respiratory muscles and pelvic muscles. At the same time, the patient smoothly tilted his neck and head forward and backward with periodic turns to the right and left. Total daily number of exercises was performed in fractions of up to five sessions per day. Next, the patient was prescribed to perform flexion and extension of the lower extremities (without taking the legs off the bed) fractionally in the supine position with the frequency described above. Their total number was increased from 50 to 100 times a day. Then the patient was

Table 2. Initial parameters of lipid metabolism and ET in healthy individuals and LLT-resistant IHD patients (M ± *m*) with ongoing maintenance pharmacotherapy

Lipid metabolism parameter	Healthy individuals	Patients of main group	Patients of control group	p^2
TC, mMol/L	5.26 ± 0.12	8.06 ± 0.11**	8.01 ± 0.10**	NS
HDL-C, mMol/L	1.85 ± 0.06	1.14 ± 0.05**	1.15 ± 0.05**	NS
LDL-C, mMol/L	3.13 ± 0.16	5.31 ± 0.14**	5.25 ± 0.14**	NS
AC	1.84 ± 0.07	6.47 ± 0.44**	6.40 ± 0.38**	NS
TG, mMol/L	1.14 ± 0.11	3.59 ± 0.21**	3.52 ± 0.19**	NS
ET, kgm /min	658.0 ± 35.4	408.6 ± 27.1**	424.1 ± 30.8**	NS

Note: ** — statistically significant differences between initial values in patients compared to values in healthy individuals ($p^1 < 0.01$); NS — differences between OG initial values and CG initial values are statistically insignificant (p^2); TC — total cholesterol; HDL-C — high-density lipoprotein cholesterol; LDL-C — low-density lipoprotein cholesterol; AC — atherogenic coefficient, TG — triglycerides; ET — exercise tolerance.

Table 3. Dynamics of lipid metabolism parameters and ET in IHD patients resistant to LLT in MG and CG (M ± *m*)

Lipid metabolism parameter	Patients of MG, initial values	Patients of MG, values after LG and UTG	Patients of CG, initial values	Patients of CG, values after PT	*p*
TC, mMol/L	8.06 ± 0.11	5.83 ± 0.10**	8.01 ± 0.10	7.87 ± 0.12	NS
HDL-C, mMol/L	1.14 ± 0.05	1.46 ± 0.04**	1.15 ± 0.05	1.21 ± 0.05	NS
LDL-C, mMol/L	5.31 ± 0.14	3.47 ± 0.14**	5.25 ± 0.14	5.22 ± 0.13	NS
AC	6.47 ± 0.44	3.22 ± 0.19**	6.40 ± 0.38	6.26 ± 0.37	NS
TG, mMol/L	3.59 ± 0.21	1.81 ± 0.11**	3.52 ± 0.19	3.54 ± 0.20	NS
ET, kgm /min	408.6 ± 27.1	501.7 ± 27.6**	424.1 ± 30.8	439.7 ± 31.7	NS

Note: ** — $p < 0.01$; NS — difference between values before and after treatment is not significant; TC — total cholesterol; HDL-C — high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; AC — atherogenic coefficient, TG — triglycerides; ET — exercise tolerance.

prescribed squatting — he leaned forward, rested his hands on the corresponding knee joints, squatted smoothly and rose in the reverse order. Squatting was performed in portions. The total number of squats was from 10 to 50 times a day.

Data statistical processing was performed using Microsoft Excel and SPSS Statistics. Values of arithmetic mean (M), standard deviation (δ), and arithmetic mean error (m) were determined. For estimating the statistical significance of differences, *t*-test was used. P-values less than 0.05 were considered to be statistically significant.

RESULTS

In patients of both groups, despite taking LLD, dyslipidemia was originally reported: hypercholesterolemia, increased LDL-C, decreased HDL-C, increased AC and triglyceridemia. Based on the baseline VEM test data, a significant decrease in ET compared to norm values was reported for both groups, indicating a decrease in the reserve capacity of the cardiovascular system (Table 2).

The comparative evaluation of the initial parameters of blood lipid spectrum and ET in patients of the two groups showed that differences in the levels of total cholesterol, HDL-C, LDL-C, AC, TG as well as ET were statistically insignificant (Table 2). The two groups were therefore initially comparable in terms of the mentioned parameters.

Thus, the examined patients of MG and CG had confirmed LMD resistant to lipid-lowering pharmacotherapy and decreased ET indicating the impaired CV reserve.

The obtained data (Table 3) demonstrated a significant lipid lowering effect of the treatment in MG patients who received treatment with ILBI and external LT in the infrared range as well as physical rehabilitation in the form of UTG. In this group, we found a significant decrease in the levels of total cholesterol (by 27.7%, $p < 001$), LDL-C (by 34.7%, $p < 001$), AC (by 50.2%, $p < 001$) and TG (by 49.6%, $p < 001$), and a significant increase in HDL-C level (by 28.1%, $p < 001$). These positive changes in MG patients were accompanied by a significant increase in ET. At the same time, no positive dynamics of the lipid profile parameters and ET were observed in CG patients (Table 3).

Thus, using the therapeutic complex including percutaneous and intravenous LT in combination with UTG physical rehabilitation in IHD patients with LMD refractory to LLT contributes to significant improvements in blood lipid parameters and increase of ET. The developed treatment complex including ILBI and external infrared LT in combination with UTG physical rehabilitation is recommended for use in IHD patients with LMD resistant to LLT.

DISCUSSION

A long-term increase in the level of atherogenic lipids in blood is believed to contribute to progressing atherosclerotic vascular damage while the decrease can inhibit the development of the pathological process and improve the course and prognosis of the disease. The examined patients of MG and CG had significant impairments in blood lipid metabolism resistant to LLT and decreased ET indicating the impaired CV reserve. This confirms that IHD patients with LMD resistant to LLT also have cellular, tissue and microcirculatory disorders that cannot be cured by using pharmacotherapy only. For their proper treatment, UTG is recommended for use as well.

Blood-to-cell metabolism occurs at the level of capillaries. In earlier studies it was shown that microcirculation damage in atherosclerosis and IHD is systemic. The number of functioning capillaries in atherosclerosis and IHD was found to be significantly lower compared to the norm, i.e. the total exchange surface of capillaries was reduced significantly [33].

Capillaries form the basis of cardiovascular reserve. Normally, about 20% of the capillary network is functioning at rest while the remaining 80% is in a reserve state. This means that the heart, due to its pumping function, maintains blood supply of only 20% of the exchange surface of capillaries. To maintain the performance of 80% of capillary exchange surface area, proper levels of physical activity are required [1–3].

In case of significant decrease in the total exchange surface of capillaries, not only the level of cholesterol but also some other substances in blood are elevated including insulin and glucose levels (type II diabetes mellitus); blood viscosity and clotting, blood pressure, peripheral vascular resistance may increase as well. The limited total exchange surface of capillaries results in insufficient cholesterol supply in cells and elevated cholesterol levels in blood accordingly [1, 3].

Increased cholesterol level and dyslipoproteidemia are believed to result from the reduction of the total exchange surface of capillaries due to a decrease in a number of not only reserve capillaries (80%) but also active capillaries (total exchange surface of capillaries decreases by 3 or 4 times). Low-density lipoproteins cannot pass through the limited exchange surface of capillaries sufficiently so their level in the blood increases. Cholesterol acts as essential building blocks of the plasma membranes. Insufficient supply of cholesterol to cells slows down the growth and development of young and stem cells leading to a slowdown of regenerative processes accordingly. Low-density lipoproteins pass through the capillary network releasing cholesterol to body cells and converting into HDL-C. High-density cholesterol in the liver is used for

production of bile acids entering the duodenum as part of bile for food digestion. In patients with atherosclerosis and IHD with manifestations of dyslipoproteinemia, on the one hand, have less high-density cholesterol, and on the other hand, they are used for synthesis of bile acids excreted as part of bile into the duodenum. Therefore, in atherosclerosis and IHD the level of atherogenic cholesterols is increased while the level of anti-atherogenic cholesterols is decreased. Consequently, restoring and maintaining the reserve and total exchange surface of capillaries using the unloading exercises has an important pathogenetic significance in case of atherosclerosis, IHD, lipid metabolism disorders, type II diabetes mellitus, arterial hypertension, circulatory insufficiency, etc. [1–3].

We consider it undesirable to overload the liver with high doses of LLD which when taken for a long time, may cause liver cell damage and other side effects, requiring biochemical control of liver cell functions since there is a new simple physiologic technique for regulating metabolism and blood lipid spectrum that can be used along with the maintenance PT. Many patients have various concomitant chronic diseases — in practice, 3 or 4 different groups of medications can be prescribed independently by different physicians for long-term use, and a patient sometimes should take up to 12 tablets per day that leads to polypharmacy. The treatment method developed by us can significantly reduce the pharmacological burden on patients.

Performing UTG daily helps to restore and maintain the total and reserve exchange surface of capillaries, relieve the work of the heart and keep its positive effect for a long period of time in cases of LMD resistant to LLT in IHD patients [1, 3]. In this case, cholesterol reaches the cells in sufficient quantities, the level of cholesterol and its atherogenic fractions in blood decreases and the level of anti-atherogenic fractions increases. This facilitates faster recovery and regenerative processes and slows down the damage processes [1, 3] as demonstrated in our study. Thus, we have developed the effective way to regulate cholesterol metabolism and control dyslipoproteidemia.

CONCLUSIONS

In IHD patients with LMD resistant to lipid-lowering pharmacotherapy, using laser therapy in combination with unloading therapeutic gymnastics makes it possible to correct disorders in blood lipid spectrum — the levels of high-density lipoprotein cholesterol are increased while the levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides are decreased significantly. The atherogenic coefficient values are decreased as well in case of such treatment. These positive changes favorably affect the functional state of patients as evidenced by the increase in exercise tolerance serving as a marker of CV reserve recovery.

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RESULTS OF ULTRASOUND SCREENING FOR HIP DYSPLASIA IN INFANTS

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Hip dysplasia (HD) represents the congenital underdevelopment of the hip joint (HJ) being the most common orthopedic problem of newborns having the prevalence of 5–20%. Late HD detection is the main cause of coxarthrosis in young adulthood. The study was aimed to assess the results of ultrasound screening for HD in infants. The study involved 860 full-term infants aged 1–3 months (446 boys (51.9%) and 414 girls (48.1%)). All newborns underwent ultrasound imaging of the hip joint at the age of 1 month and the follow-up examination at the age of 3 months (Graf method). The χ^2 test and $p < 0.05$ were used to compare the data. In their first year of life, 685 newborns (79.7%) had joints of normal or transitory shape, 161 (18.7%) showed physiological immaturity and 14 (1.6%) showed the HJ abnormality; the HJ immaturity and abnormality were more prevalent in girls (113 cases (26.3%)) than in boys (62 cases (13.9%)). The relationship between the breech presentation and the likelihood of developing HD was revealed ($p < 0.001$). Spontaneous improvement by the age of 3 months took place in the majority of infants having the ultrasound signs of HD, the rate of normal HJ increased from 79.8 to 94.5%. Ultrasound screening is an effective method allowing one to detect HD starting from the first days of the child's life. The risk factors of HD are still female sex and breech presentation, regardless of the number of births. Spontaneous improvement following prescription of relaxing massage occurs in the majority of children.

Keywords: hip dysplasia, ultrasound screening of newborns, hip sonography, congenital hip dislocation

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Compliance with the ethical standards: the study was conducted in accordance with the Order of the Ministry of Health of the Russian Federation dated 28.04.2007 No. 307 "On the Standard of Dispensary (Preventive) Observation of a Child during His/Her First Year of Life". Parents submitted the informed consent to ultrasound. No approval by the Ethics Committee was required.

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РЕЗУЛЬТАТЫ УЛЬТРАЗВУКОВОГО СКРИНИНГА ДИСПЛАЗИИ ТАЗОБЕДРЕННЫХ СУСТАВОВ У ДЕТЕЙ ГРУДНОГО ВОЗРАСТА

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Дисплазия тазобедренного сустава (ДТБС) — врожденное недоразвитие тазобедренного сустава (ТБС), наиболее распространенное ортопедическое заболевание новорожденных, встречающееся с частотой 5–20%. Поздно выявленная ДТБС — основная причина развития коксартроза в молодом возрасте. Целью исследования было провести анализ результатов ультразвукового скрининга ДТБС у детей грудного возраста. В исследование вошли 860 доношенных детей возрастом 1–3 месяцев (446 мальчиков (51,9%) и 414 девочек (48,1%)). Всем новорожденным выполняли ультразвуковой скрининг ТБС в возрасте 1 месяц и контрольное исследование в 3 месяца (методика по Графу). Для сравнения данных использовали критерий (χ^2) и $p < 0,05$. В первый месяц жизни у 685 (79,7%) новорожденных суставы были нормальной или транзиторной формы, у 161 (18,7%) выявлена физиологическая незрелость и у 14 (1,6%) — патология ТБС, у девочек незрелость и патология ДТБС встречалась чаще — в 113 (26,3%) случаев, чем у мальчиков — 62 (13,9%). Выявлена связь между тазовым предложением плода и вероятностью развития ДТБС ($p < 0,001$). К трем месяцам у большинства детей с УЗИ-признаками ДТБС произошло спонтанное улучшение, показатели нормальных ДТБС выросли с 79,8 до 94,5%. УЗИ-скрининг — эффективный метод, который позволяет выявлять ДТБС с первых дней жизни ребенка. Факторами риска развития ДТБС остаются женский пол и ягодичное предлежание, вне зависимости от числа родов. У большинства детей улучшение происходит спонтанно или при назначении расслабляющего массажа.

Ключевые слова: дисплазия тазобедренных суставов, ультразвуковой скрининг новорожденных, сонография тазобедренных суставов, врожденный вывих бедра

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Соблюдение этических стандартов: исследование проводили в соответствии с Приказом МЗ РФ от 28.04.2007 № 307 «О стандарте диспансерного (профилактического) наблюдения ребенка в течение первого года жизни». На проведение УЗИ родители дали добровольное информированное согласие. Разрешение этического комитета не требовалось.

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Hip dysplasia (HD) represents the congenital underdevelopment of the hip joint (HJ) associated with deformities of the articular ends of bones manifesting themselves in alterations of the shape and depth of the acetabulum, neck shaft angle, and proximal femur. HD is among the most common orthopedic problems of newborns, it constitutes 12–22% of all musculoskeletal dysplasias. The prevalence of this disorder in various populations varies between 50–200 cases per 1000 newborns (5–20 %) [1]. Developmental abnormalities of the HJ resulting from the delayed diagnosis or inadequate conservative treatment of HD are the main cause of developing dysplastic coxarthrosis in young adulthood (10–60% of cases) [2]. There is a problem of defining and clarifying epidemiological data on this disorder that is associated with the lack of official universal criteria and classification concept of HD [3]. In some children with the physiologically immature HJ, dysplasia persists after 3 months (Graf type 2b joint), without decentered femoral head or any clinical manifestations. The treatment tactics for such patients is still a matter of debate [4, 5].

The study was aimed to assess the results of the orthopedic screening of infants performed using ultrasound imaging.

METHODS

The study was based on the monitoring data of 860 children aged 1–3 months attached to the Children's City Polyclinic № 5 in the city of Vladivostok, who underwent ultrasound screening of the HJ at the age of 1 month (in accordance with the Graf method) during preventive medical examination between December 2021 and August 2023. Re-examination by pediatric orthopedic traumatologist and the follow-up ultrasound imaging (performed with the Mindray DC-70 Pro expert class ultrasound system (Mindray; China)) of the HJ were performed at the age of 3 months. In the initial group of 860 individuals, a total of 791 children were examined at the age of 3 months; other children left the territory of attachment to the clinic. Both male and female full-term infants (gestational age 38–42 weeks) were included in the study: 446 boys (51.9%) and 414 girls (48.1%). The infants, whose gestational age was under 37 weeks, and post-term infants (gestational age 42 weeks) were excluded from the study. A total of 822 children (95.6%) had cephalic presentation, 38 children (4.4%) had breech presentation (patients were not divided into groups based on the breech presentation type). It was noted, that breech presentation was more common in girls (25 (6.0%)), than in boys (13 (2.9%)). Primiparity took place in 335 cases (38.9%), the second birth took place in 393 cases (45.7%), the third or more birth took place in 132 cases (15.4%). The data were presented as absolute values and percentages. Pearson's chi-squared test (χ^2) was used to compare the data. The differences were considered significant at $p < 0.05$. The STADIA 8.0 universal statistical software package (A.P. Kulaichev; Russia) was used for data processing.

RESULTS

When performing examination, we assessed hyperechoic bone structures (bony part of the acetabular roof, external bony prominence, external part of the ilium, femur) and hypoechoic structures (femoral head, limbus, triradiate cartilage). Relative positions of the joint components were determined based on the bony roof angle (α angle) and the angle formed by the cartilaginous roof (β angle). According to the Graf classification, four types of HJ are distinguished based on the angles and the femoral head position [3, 6]:

1 a — mature hip (α angle > 60°, β angle < 55°);

1 b — transitory hip (α angle > 60°, β angle > 55°);

2 a — physiological immaturity under the age of 3 months (α angle = 59–50°, β angle > 55°);

2 b — HD (shallow acetabulum, α angle = 59–50°, β angle > 55°) in children over the age of 3 months;

2 c — severe HD, preluxation (α angle = 43–49°, β angle 56–77°);

type D — severe HD, slight decentralization;

3 a,b — decentered HJ (subluxation) (α angle < 43°, β angle < 43°);

4 — severe HJ dislocation.

In our study, 685 newborns (79.7%) had type 1a,b type HJ (mature or transitory HJ) in their first month of life; physiological immaturity, type 2a HJ (flattening of the bone edge, expansion and shortening of the limbus, shallow, flattened acetabular floor) was reported in 161 children (18.7%). Abnormalities were detected in 14 newborns (1.6%). Type 2c HJ was reported in 10 cases (femoral head decentered only during functional tests, preluxation; stable and unstable joints were not distinguished), subluxation of the hip, type 3a,b HJ (decentered joint), was reported in three cases (later confirmed by clinical and radiography data). Congenital hip dislocation (type 4 HJ) was detected in one infant. This patient was referred to inpatient treatment and withdrawn from the study.

It should be noted that the HJ immaturity and HD were more common in girls (113 cases (27.3%)), than in boys (62 cases (13.9%)). Among children with breech presentation, immaturity of the HJ was detected in 18 cases (47.3%), while among children with cephalic presentation it was detected in 157 cases (13.9%). We revealed a significant correlation between the infant's female sex, breech presentation, and the likelihood of developing HD ($p < 0.001$). At the same time, there was no relationship between the detection frequency of HD in the first-born and later-born individuals ($p = 0.495$) (Table 1).

The recommended infant management algorithm was selected based on the HJ ultrasound imaging data [6]. Children with type 2a,b and 3a,b HJ (175 patients (20.3%)) underwent the course of massage; the fixing orthoses were used in individuals with the adductor contracture and decentered femoral head. The follow-up ultrasound imaging of the HJ was performed at the age of 3 months. Abnormalities (joint type 2b, 3a,b), i.e. flattening of the bone edge, expansion and shortening of the limbus, shallow, flattened acetabular floor, decentered femoral head, α angle < 59°, β angle > 55°, persisted in 48 children (6.1%) (Table 2).

DISCUSSION

Our findings are in line with the literature data: the non-physiological position of the fetus inside the uterus due to the child's legs pressed up (breech presentation), especially under conditions of oligoamnios, represents a prognostic sign and increases the likelihood of developing HD [1, 6, 7]. The fact that HP is more common in girls has been confirmed. This can be explained by the exposure to extra estrogens produced by female fetus, which contributes to the ligament laxity. At the same time, we have revealed no relationship between HD and the number of births.

The majority of children having ultrasound signs of HD show spontaneous improvement or improvement following prescription of relaxing massage [8, 9]. Thus, according to the data of our study, joints of 1a type (normal) were revealed in 743 children by the age of 3 months. The group of children with normal HJ expanded within two months: from 79.7% at the first

Table 1. Ultrasound imaging results obtained at the age of 1 month

Trait	HJ type (Graph classification)			χ^2 test and significance level (2 degrees of freedom)
	1 a, b	2 a, b	2 c; 3 a, b; 4	
Sex (abs./%)				
Boys (n = 446)	384 (86,1)	59 (13,2)	3 (0,7)	χ^2 = 24.957, p < 0.001, significant correlation
Girls (n = 414)	301 (72,7)	102 (24,6)	11 (2,7)	
Presentation (abs./%)				
Cephalic (n = 822)	665 (80.4)	147 (17.9)	10 (1.7)	χ^2 = 29.955, p < 0.001, significant correlation
Breech (n = 38)	20 (52.7)	14 (36.8)	4 (10.5)	
Birth (abs./%)				
First (n = 335)	260 (77.6)	69 (20.6)	6 (1.8)	χ^2 = 1.408, p = 0.495, non-significant correlation
Repeated (n = 525)	425 (81.0)	92 (17.5)	8 (1.5)	
Total	685 (79.7)	161 (18.7)	14 (1.6)	860 (100)

Table 2. Comparison of ultrasound imaging results obtained at the age of 1 month and 3 months

Months of life	HJ type (Graph classification)		
	1 a, b	2 a, b	2 c, 3 a, b; 4
1 month (n = 860)	685 (79.7)	161 (18.7)	14 (1.6)
3 months (n = 791)	743 (93.9)	45 (5.7)	3 (0.4)

Note: χ^2 = 72.036 (2 degrees of freedom), p < 0.001, significant correlation.

examination to 93.9% at the follow-up examination conducted at the age of 3 months. Prescription of prolonged immobilization to such children can cause necrosis of the femoral head, while prescription of the courses of intense massage can increase joint instability.

HD is divided into hip preluxation, subluxation, dislocation based on the severity of symptoms and prognosis. Congenital hip displacement is observed in 0.1–0.4% of newborns, while preluxation and subluxation are 10 times more prevalent [10, 11]. In our study, congenital hip displacement was diagnosed in one newborn (0.1%). There is a possibility that children with congenital hip displacement are transferred to the specialized orthopedic unit from maternity unit. That is why these children were not included in our statistics.

The HD diagnosis is based on a combination of clinical symptoms: Barlow provocative maneuver, the “click” sign (Ortolani sign), asymmetry of femoral and gluteal folds, limited hip abduction, lower limb shortening, excessive rotational movements. The diagnostic value of the symptoms detected in young children, except for clinical tests for instability and limb shortening, is 50–80%, since these symptoms are rather common in children showing no HJ underdevelopment. It is not always possible to notice the differences in the limb length in newborns, and provocative tests require good skills. The diagnostic radiography is highly informative, however, X-ray diagnosis of HP abnormalities in children in their first months of life is hampered by the fact that the HJ skeletal system of a newborn consists partially of the cartilage tissue. Ultrasound imaging of the HJ in newborns has none of these problems, that is why it is being used since 1980s [1, 4, 9, 12]. The method makes it possible to diagnose the most prevalent acetabular HD that is found in 62% of cases. Stable proximal femoral dysplasias (abnormalities not resulting in the femoral head decentering — coxa valga and coxa vara, femoral neck shortening, rotational dysplasia) rarely respond to conservative therapy and require surgical treatment. In young children with acetabular dysplasia, the HJ deformity is successfully corrected through adequate conservative treatment (massage, orthopedic positioning devices, closed reduction of dislocation). If the disorder is detected in children under the age of 3 months, it possible to achieve good outcome in 97% of cases [11].

However, there are certain difficulties related to interpretation of sonography results. Orthopedic traumatologists follow a common treatment tactics for congenital hip subluxation and displacement (correspond to the Graf HJ types 3a,b–4) involving early orthopedic treatment, while the management algorithm for infants with the HJ type 2b, specifically treatment duration, is still a matter of debate [1, 6, 12]. Persistent minor alteration of the head and acetabular arc without apparent joint instability can later result in orthopedic disorder of the lower limbs. In our opinion, in this situation, when selecting treatment tactics, priority should be given to the data obtained during radiographic diagnosis and follow-up examinations of the child.

CONCLUSIONS

Sonography in infancy is a highly informative method to diagnose HD due to high prevalence of the disorder among newborns, low informational content of radiographic methods, and ambiguity of the examination data interpretation. Analysis of the results of ultrasound orthopedic screening of infants has shown that female sex and breech presentation remain the risk factors of HD development in children, regardless of the number of births. The majority of children with ultrasound signs of HD show spontaneous improvement or improvement following prescription of relaxing massage. In case of persistent minor alterations of the HJ without apparent joint instability, the data of radiographic diagnosis and follow-up examinations of the child become a priority for selection of treatment tactics.

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EXPERT ASSESSMENT OF THE REQUIREMENTS FOR ASSIGNING QUALIFICATION GRADES TO PHYSICIANS

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Shortcomings of the categorization system include weakness of the normative regulation oriented towards the outdated ideas about the qualification and formal features of professional development. It is often proposed to objectify measuring the professional competence of the subjects evaluated in order to improve the categorization system. The study was aimed to test the qualification requirements of physicians for the relationship with their qualification and the possibility of accurate full-fledged measurement of those in the evaluated subjects. We performed expert assessment of 22 requirements for grades approved by the Order of the Ministry of Health of the Russian Federation dated 31 August 2023 No. 458n relative to four items: their relationship with the physician's qualification, feasibility of measurement (usability), relationship with the competence of the evaluation commission member (objectivity), possibility of determining the extent of the knowledge, abilities, skills required for each qualification grade. Assessment involving the use of the Stapel rating scale ("–5" to "+5") was performed by seven experts. The sums of scores by items were as follows: relationship with qualification — 477, usability — 316, objectivity — –662, grade — –699. There are significant differences between the scores reported for all the requirements and pairs of all items ($p \leq 0.0001$), except the objectivity–grade pair ($p = 0.103$). The total of the scores reported for the majority of requirements is negative due to the lowest possible scores of objectivity and grade. The experts believe that none of the qualification requirements approved by the Order enables accurate full-fledged determination of physician's qualification during evaluation.

Keywords: categorization, qualification grade, professional development

Author contribution: Misharin VM — research coordination, literature review, data analysis and interpretation; Kochubey AV — concept, design, statistical analysis, conclusions, manuscript writing.

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ЭКСПЕРТНАЯ ОЦЕНКА ТРЕБОВАНИЙ ДЛЯ ПРИСВОЕНИЯ КВАЛИФИКАЦИОННОЙ КАТЕГОРИИ ВРАЧАМ

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К недостаткам системы аттестации относится слабость нормативного регулирования, ориентированного на устаревшие представления о квалификации и формальные признаки профессионального развития. Для совершенствования системы аттестации часто предлагают объективизацию измерения профессиональной компетентности аттестуемых. Целью исследования было оценить требования к квалификации врачей на предмет их связи с квалификацией, возможности точного и полноценного измерения у аттестуемых. Проведена экспертная оценка 22 требований к категориям, утвержденным приказом Минздрава России от 31 августа 2023 г. № 458н, относительно четырех полей: их связи с квалификацией врача, выполнимости измерения (практичности), зависимости от компетентности члена аттестационной комиссии (объективности), возможности установить степень развития требуемых знаний, умений, навыков для каждой квалификационной категории (градации). Оценку проводили семь экспертов по шкале Стейпла от «–5» до «+5». Сумма баллов по полям: связь с квалификацией — 477, практичность — 316, объективность — –662, градация — –699. Имеется значимая разница оценок по всем требованиям и парам всех полей ($p \leq 0{,}0001$), кроме пары «объективность» и «градация» ($p = 0{,}103$). Общая сумма баллов большинства требований отрицательна из-за максимально низких оценок объективности и градации. По мнению экспертов, ни одно из утвержденных Приказом требований к квалификации не позволяет точно и полноценно определить квалификацию врача при аттестации.

Ключевые слова: аттестация, квалификационная категория, профессиональное развитие

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In an effort to ensure continuous professional development of healthcare professionals, national public health systems create various institutions to stimulate or motivate medical personnel to upgrade their skills [1–3]. The domestic public health system is no exception. Currently, the institutions of certification, categorization, continuing medical education, independent qualification evaluation, internal categorization, are more or less active in our country [4–7]. Functioning of all the above institutions is regulated by the statutory acts of various levels. In particular, categorization of physicians is regulated by the Order of the Ministry of Health [4] updated in 2023 to bring the procedure of assigning qualification grades in line with the newly created categorization and continuing medical education systems.

It is no secret that the domestic institution for categorization of healthcare professionals and pharmacists has been the subject of criticism: overlapping of tasks with the new qualification evaluation institutions was reported, and the gaps in the normative regulation oriented towards the outdated ideas about the qualification and formal characteristics of

professional development were disclosed [8–10]. It is clear that the use of formal professional development characteristics as evaluation criteria has led to the categorization weakness in terms of qualification measurement, which is confirmed by the data of expert assessment of the quality of medical care provided by individuals, who have passed categorization [11]. Furthermore, healthcare professionals don't want to give up the categorization institution, but see the need to change it in accordance with the current understanding of physician's professional development [12].

It should be noted that creating incentives for passing categorization and objectivization of measuring professional competence of evaluated individuals have been leading among proposals to improve the categorization system for healthcare professionals for many years [13]. It is likely that these proposals are reflected in the current procedure of assigning qualification grades in the form of the requirements for knowledge, abilities, skills established for qualification grades of certain levels.

In this regard, the study was aimed to test the qualification requirements of physicians for the association with their qualification and the possibility of accurate full-fledged measurement of those in the evaluated physicians.

METHODS

To achieve the goal, we performed expert assessment of the requirements for assigning various qualification grades established by the Order of the Ministry of Health of the Russian Federation dated 31 August 2023 № 458n " On Approval of the Procedure and Timing for Healthcare and Pharmaceutical Professionals to Undergo Categorization to be Assigned a Qualification Grade" (hereinafter, the Order). The expert assessment was performed based on four items:

– association with the physician's qualification, i.e. determining whether the required knowledge, abilities, and skills are direct (not formal) signs of professional qualification;

– usability, i.e. feasibility of measuring the required knowledge, abilities, and skills;

– objectivity, i.e. determining whether the measurement results depend on the instruments used for assessment, competence or personal preferences of the expert panel members;

– grade, i.e. determining whether it is possible to accurately determine the extent of the knowledge, abilities, and skills required for each qualification grade.

Expert assessment of all items was performed using the conventional Stapel rating scale ("–5" to "+5") without a null-value, where "–5" meant that the requirement did not ensure the association with the physician's qualification, usability, objectivity, grade, while "+5" meant that the requirement guaranteed the association with the physician's qualification, usability, objectivity, grade.

All the experts were provided the guidelines on understanding the items and scales. They were also given an evaluation sheet with the described above Stapel rating scale containing the list of 22 requirements for knowledge, abilities, and skills specified in the Order. Expert assessment was moderated by the authors. The experts did not communicate with each other.

The group of experts consisted of seven people. Criteria for selection of experts: graduate in medicine, academic degree of Doctor of Medical Sciences, medical experience exceeding 10 years, experience of scientific and pedagogical activity of at least 7 years, experience in categorization commissions, experience in expert panels. Exclusion criteria: age over 60 years, formally ended medical, scientific and pedagogical professional activity. The experts' average medical experience was 24.0 ± 2.0 years, and their average experience of scientific and pedagogical activity was 13.4 ± 2.6 years.

Statistical processing of the expert assessment results was performed using the IBM SPSS software. We calculated the sums of scores for four items and 22 requirements. Given the fact that the majority of variables had non-normal distributions after applying the Kolmogorov–Smirnov test ($p \le 0.0001$), we applied the Mann–Whitney U test to confirm the differences in expert assessment (pairwise for four items and distinct requirements) and the Kruskal–Wallis test to prove the differences in expert assessment (for all requirements).

RESULTS

The sums of expert assessment scores by items were as follows: association with qualification — 477, usability — 316, objectivity — –662, grade — –699. The Mann–Whitney U test calculation revealed significant differences between variables for pairs of all items ($p \le 0.0001$), except the objectivity–grade pair ($p = 0.103$).

The Table provides the sums of the experts' scores for each requirement (by item) and the total scores. The Kruskal–Wallis test calculation showed that the experts assessed the required knowledge, abilities, and skills by four items differently ($p \le 0.001$).

In experts' opinion, the requirement for the ability to estimate the data of specific assessment methods to make the diagnosis, for theoretical knowledge and practical skills in the field of professional activity, for applying the prevention, diagnosis, treatment, and rehabilitation methods used in the world's and domestic medicine showed the equally strong associations with qualification ($0.073 \le p \le 1.0$). Furthermore, these associations were significantly stronger than that of other requirements ($0.001 \le p \le 0.026$).

The experts rated higher usability of the requirement for the ability to estimate the data of specific assessment methods to make the diagnosis, than that of other requirements ($0.001 \le p \le 0.004$). Objectivity of the requirement for using medical products was rated higher, than that of other requirements ($p = 0.001$). In experts' opinion, objectivity of other requirements was the same ($0.383 \le p \le 1.1$). All the requirements had an equally low grade ($0.710 \le p \le 1.0$), except the requirements for professional experience.

Three requirements had positive total scores (in descending order): requirements for the experience, for using medical products, and for the ability to estimate the data of specific assessment methods to make the diagnosis. The requirement for the experience was assigned the highest usability, objectivity, and grade scores, but the lowest "association with qualification" scores. The requirement for the ability to estimate the data of specific assessment methods to make the diagnosis, on the contrary, was assigned the highest association with qualification and usability scores, but the lowest objectivity and grade scores. The requirement for using medical products was assigned positive association with qualification, usability, and objectivity scores, but the lowest grade scores. The total expert assessment score of the requirement for using medical products (for all items) is significantly higher, than that of other requirements ($0.0001 \le p \le 0.002$), except the requirements for professional experience.

DISCUSSION

The study has shown that the experts consider more than a half of the required knowledge, abilities, and skills as the

Table. Expert assessment of requirements for qualification

№	Requirements	Sum of scores				Total
		Association	Usability	Objectivity	Grade	
1	Experience	–32	35	35	35	73
2	Theoretical knowledge — in the field of professional activity	31	27	–33	–35	–10
3	— in the field of related disciplines	30	27	–35	–35	–13
	Practical skills					
4	— in the field of professional activity	31	29	–35	–35	–10
5	— in the field of related disciplines	30	29	–35	–35	–11
	Using the methods applied in world's medicine					
6	— prevention	31	20	–35	–35	–19
7	— diagnosis	31	20	–35	–35	–1–
8	— treatment	31	20	–35	–35	–19
9	— rehabilitation	31	20	–35	–35	–19
	Using the methods applied in domestic medicine					
10	— prevention	31	20	–35	–35	–19
11	— diagnosis	31	20	–35	–35	–19
12	— treatment	31	20	–35	–35	–19
13	— rehabilitation	31	20	–35	–35	–19
14	Using the medical products	19	26	1	–35	11
	Skills of assessing performance indicators					
15	— quantitative	8	27	–35	–35	–35
16	— qualitative	9	27	–35	–35	–34
17	Completing the work report	–2	–9	–35	–35	–81
18	Using scientific and technical information	27	–22	–35	–35	–65
19	Part in resolving tactical issues	16	–21	–35	–35	–75
	Using scientific and technical information to resolve					
20	— tactical issues	14	–28	–35	–35	–84
21	— strategic issue	13	–26	–35	–35	–83
22	Skill of estimating the data of specific assessment methods to make the diagnosis	35	35	–35	–34	1

most strongly associated with professional qualification and measurable. However, the experts believe that it is impossible to ensure objectivity (including independence from the expert panel member's competence) for the largest part of the required knowledge, abilities, and skills. Furthermore, the experts see no possibility to accurately determine the extent of the knowledge, abilities, and skills required for each qualification grade for the vast majority of qualification requirements.

Two requirements in the "association with qualification" item were assigned negative scores: experience and completing the work report. It is important to note that experience has long been considered as a formal characteristic of professional development [14]. The consensus among experts confirms that the length of service does not guarantee the physician's high qualification.

Poor expert assessment of the association of the requirements for completing the work report, requirements for the skills of assessing quantitative and qualitative performance indicators with qualification is interesting amidst the data on poor physicians' competence in completing the work reports and dealing with statistical data [11].

As for usability of the requirements, i.e. feasibility of measuring the required knowledge, abilities, and skills, the experts consider only five requirements out of 22 as not measurable. It is worth mentioning that the experts have noted weak association of certain requirements with qualification. These are requirements for completing the work report, using scientific and technical information, part in resolving tactical issues, using scientific and technical information to resolve tactical issues, using scientific and technical information to resolve tactical and strategic issues.

The authors see two limitations affecting the study results: the experts' personal attitude towards the categorization system, from hostility to sympathy, and the shortcomings of the Stapel rating scale represented by ambiguity in understanding the scale divisions by the respondents.

CONCLUSIONS

None of the qualification requirements approved by the Order enable accurate full-fledged determination of the evaluated individual's qualification. The main shortcomings of the established requirements are as follows: absolute dependence of assessment on the expert panel member's competence and impossibility to determine the extent of knowledge, abilities, and skills required for specific qualification grade. The experts believe that the requirement for experience, that has been assigned the highest positive objectivity, grade, and usability scores by the experts, does not reflect the physician's professional qualification.

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ASSESSMENT OF THE IMPACT OF RETROCEREBELLAR CYSTS IN THE BRAIN ON THE CEREBROSPINAL FLUID SYSTEM AS A CRITERION OF FITNESS FOR FLIGHT

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Retrocerebellar cysts of the brain represent the aspect that is important for determination of fitness for flight. The study was aimed to assess their impact on the pilot performance by conducting comparative analysis of MRI data of the first-year cadets and experienced pilots. We assessed the prevalence of retrocerebellar cysts among cadets and pilots, conducted non-contrast brain MRI, and compared the major academic and physical performance indicators, along with the results of professional psychological screen. The prevalence of retrocerebellar cysts among first-year cadets was 8.2%. High prevalence of asymptomatic retrocerebellar cysts among experienced pilots was revealed (two cases out of five). The intergroup comparison of indicators makes it possible to draw a conclusion about probable minor impact of such changes on fitness for flight. Further research is required to clarify the mechanisms underlying the impact of retrocerebellar cysts on the pilot performance and develop appropriate guidelines for medical boards.

Keywords: military medical examination, cerebrospinal fluid system, neuroimaging, cadets, pilots

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Compliance with the ethical standards: the study was compliant with the principles of the Declaration of Helsinki.

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ОЦЕНКА ВЛИЯНИЯ РЕТРОЦЕРЕБЕЛЛЯРНЫХ КИСТ ГОЛОВНОГО МОЗГА НА ЛИКВОРНУЮ СИСТЕМУ КАК КРИТЕРИЙ ГОДНОСТИ К ЛЕТНОЙ РАБОТЕ

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Ретроцеребеллярные кисты головного мозга представляют собой важный аспект для определения пригодности к летной работе. Целью исследования было оценить их влияние на летную работу через сравнительный анализ данных МРТ курсантов первого курса и летчиков с опытом. Были выполнены анализ встречаемости ретроцеребеллярных кист у курсантов и летчиков, проведение МРТ ГМ без контрастного усиления, сравнительное исследование основных академических и физических показателей, а также результатов профессионального психологического отбора. Среди курсантов первого курса встречаемость ретроцеребеллярных кист составила 8,2%. У летчиков с опытом выявлена высокая частота бессимптомных ретроцеребеллярных кист (в двух случаях из пяти). Сравнение показателей между группами позволяет сделать выводы о возможном незначительном влиянии данных изменений на годность к летной работе. Дальнейшие исследования необходимы для уточнения механизмов влияния ретроцеребеллярных кист на летную деятельность и разработки соответствующих рекомендаций для медицинских комиссий.

Ключевые слова: военно-врачебная экспертиза, ликворная система, нейровизуализация, курсанты, летчики

Вклад авторов: С. В. Горнов — участие в научном дизайне, формирование идеи, проведение исследования; Д. В. Карпенко — редактирование текста; В. В. Горнов — анализ и интерпретация полученных данных, критический пересмотр черновика рукописи; В. Г. Коломийцев — написание исходного текста, сбор данных, разработка программного обеспечения; Р. В. Еселевич — итоговые выводы; И. В. Бурова — определение концепции работы, составление черновика рукописи, утверждение окончательного варианта статьи, ответственность за целостность всех частей статьи; Е. А. Литвиненко — разработка методологии; Р. А. Крупа — оформление рукописи.

Соблюдение этических стандартов: клиническое исследование проведено в соответствии с принципами Хельсинкской декларации.

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In 2022, the Decree of the Government of the Russian Federation No. 565 "About approval of the Regulations on military-medical examination" dated 04.07.2013 was amended [1]. According to the document, brain MRI during the medical flight expert commission (VLEK) has first become mandatory for the citizens studying at the military educational institutions responsible for training of flight personnel of the state aircraft.

The analysis of the air force cadets' first quantitative brain MRI results revealed high prevalence of various cerebrospinal fluid system alterations [2] representing the criterion for being unfit for flight training based on VLEK [1]. The data on the prevalence of alterations in the cerebrospinal fluid system of the brain among cadets were acquired during the study. That is why VLEK was given a task to determine the cadet's category of fitness for further flight training.

All the non-neoplastic abnormalities and/or variants of the central nervous system (CNS) development showing no clinical manifestations or functional impairment represent the criterion of unfitness for flying duties used when conducting VLEK, regardless of the type of aircraft [1].

The study was aimed to assess the impact of retrocerebellar cysts in the brain on the pilot's performance based on the comparative analysis of the brain MRI data of first-year air force cadets and air force pilots, who had successfully finished their flight duties.

METHODS

We used brain MRI data to assess the prevalence of retrocerebellar cysts among 348 (100%) first-year cadets studying at the Krasnodar Higher Military Aviation School of Pilots named after Hero of the Soviet Union A.K. Serov (median age was 19.0 years) during their first VLEK conducted at the 419th Military Hospital

(Krasnodar) between October 2022 and February 2023. Inclusion criteria: the first-year cadets admitted to the aviation school in 2022–2023 were included in the study. During the period when the cadets underwent MRI (October 2022 to February 2023), MRI was also performed in the retired air force pilots having no neurological complaints, who contacted the 419th Military Hospital for outpatient care. We compared retrocerebellar cysts of the cadets and five military pilots in the reserve (median age 42.8 years), who had successfully finished their flight duties and undergone brain MRI. The clinical trial was conducted in accordance with the principles of the Declaration of Helsinki.

Brain structures were assessed using the open MRI-AMICO300 scanner (AMICO; Russia) with the induction of 0.3 T. The standard brain MRI protocol was used [3]. All the acquired MRI scans (100%) were processed using the Machaon DICOM software tool.

Academic performance was assessed in accordance with the qualification requirements for professional military training of graduates.

Individual assessment of the cadets' physical fitness was performed in accordance with the thematic plan of the Department of Physical Training of the aviation school. The following parameters of physical development were distinguished: speed (100 m race), strength (pull-ups), endurance (1 or 3 km race), and agility (shuttle run 10 × 10 m).

The results of professional psychological selection (PPS) conducted at admission to the military school were used to estimate the cadets' professionally significant personality and intellectual qualities [4].

All the first-year cadets (n = 348) underwent brain MRI, no contrast agent was administered [3]. After selection, 100% of the cadets included in the study were divided into two groups based on the presence or absence of brain alterations in MRI. The group of patients having structural brain alterations consisted of 41 individuals; all the alterations found in the imaging region were assessed in order to find the most representative element that would be later defined as the object of research by the authors (retrocerebellar cysts found in 28 cadets).

Two groups of cadets were compared based on the following:

– estimates obtained when entering the aviation school and during the period of professional development (main academic disciplines and physical training);

– categories of professional psychological selection assigned when entering the aviation school;

"Adaptability" multilevel personality questionnaire designed to study adaptive capacity based on the assessment of certain psychophysiological and socio-psychological characteristics reflecting the integral features of the cadet's mental and social development.

The non-contrast-enhanced brain MRI was also performed in five patients in the group of individuals in the reserve, among them two patients underwent assessment in other medical institutions. The remoteness of assessment did not exceed two weeks from the date of assessing the main population.

RESULTS

The diagnostic tests performed as part of VLEK in 348 first-year cadets showed that the most prevalent cerebrospinal fluid system alterations were as follows: retrocerebellar cysts — 28 (8.2%), enlarged cerebrospinal fluid spaces — 3 (0.9%), asymmetry of the lateral ventricles — 3 (0.9%), septum pellucidum cyst — 3 (0.9%), internal hydrocephalus — 2 (0.6%), and temporal arachnoid cysts — 2 (0.6%). In the total sample of cadets, retrocerebellar cysts were found in 28 cases, the average cyst size was 9.5 mm (Fig. 1).

At the same time, the routine examination of five military pilots in the reserve included in the total sample revealed retrocerebellar cysts in two cases, the average cyst size was 8.1 mm. Thus, an asymptomatic retrocerebellar cyst sized 9 × 11 mm was found in patient S. aged 42 years (total flight experience exceeding 1500 h, including 800 h on the Su-24 fighter) (Fig. 2).

Comparison with the PPS data obtained at admission to the aviation school (Table 1), academic performance scores for the main disciplines and physical performance obtained throughout the entire training period (Table 2) was conducted in order to assess the CNS functional state in the cadets showing no MRI alterations (group 1) and cadets showing MRI alterations (retrocerebellar cysts) (group 2).

Pilots of group 1 were assigned PPS category 1 in 2% of cases, category 2 in 89% of cases, and category 3 in 9% of cases. Pilots of group 2 were assigned PPS category 2 in 100% of cases, which suggested functional adaptability of the cadets of group 2 (Table 1).

The average scores for the main disciplines and physical training showed no significant differences between two groups of pilots (Table 2).

The reasons for the first-year cadets' expulsion from the higher aviation school were assessed in both groups in order to compare the cadets' success (Table 3).

There were 4 cadets (10%) discharged for health reasons (due to retrocerebellar cysts) after VLEK. Twice more first-year cadets of group 2 (with retrocerebellar cysts in MRI) resigned

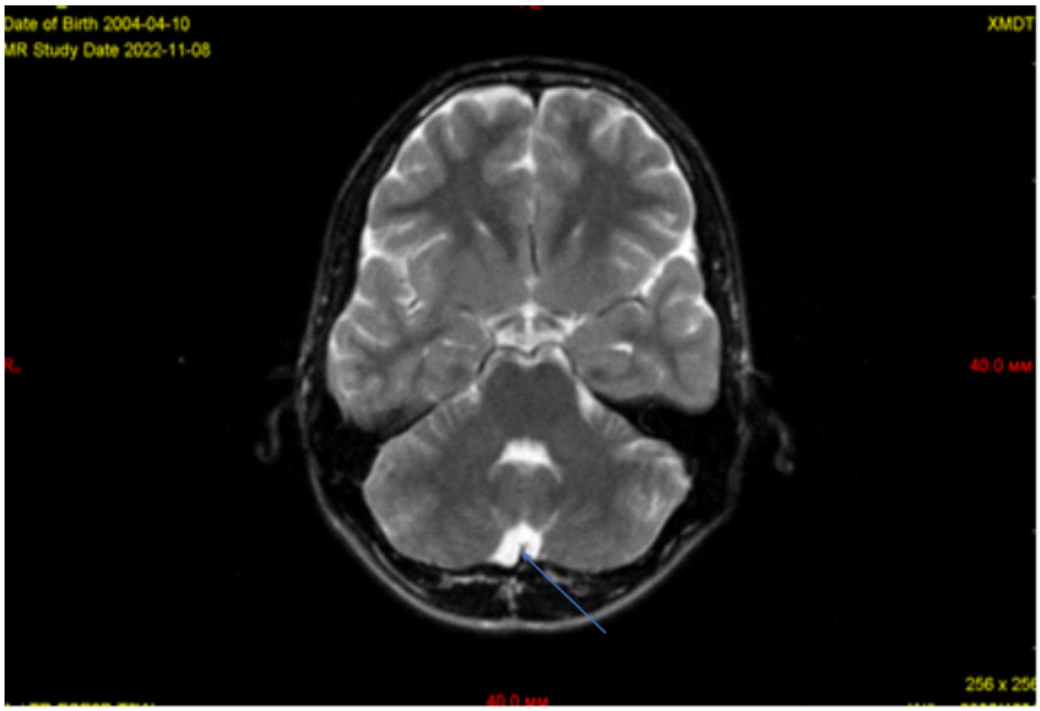

Fig. 1. T2-weighted MRI, axial slice: arachnoid spaces at the level of the cerebellar hemispheres (retrocerebellar cyst) in the form of local enlargement with the axial size up to 7 × 11 mm in the first-year cadet H. aged 20 years. This cadet, Candidate for Master of Sport in swimming, prize-winner of the Cup of the Russian Armed Forces among cadets, was recognized as unfit for flight training [1]

due to personal reasons after MRI compared to cadets showing no MRI alterations (Table 3). This was due to high psychoemotional stress and concerns for further flight career among cadets of group 2, who had undergone brain MRI.

DISCUSSION

The data on the CNS functional state (brain MRI) and personal adaptive capacity assessed during the study showed that 100% of surveyed cadets had good performance and showed no significant differences, which suggests overestimation of the impact of retrocerebellar cysts on the category of fitness for flight duties [5].

The data provided are in line with the data of the other study [6] showing excessive demands on the presence of retrocerebellar cysts and no need for mandatory dismissal from flight duties based on VLEK.

The available literature provides wide coverage of the results [7] suggesting that the size and localization of masses in the retrocerebellar region (retrocerebellar cysts) have no

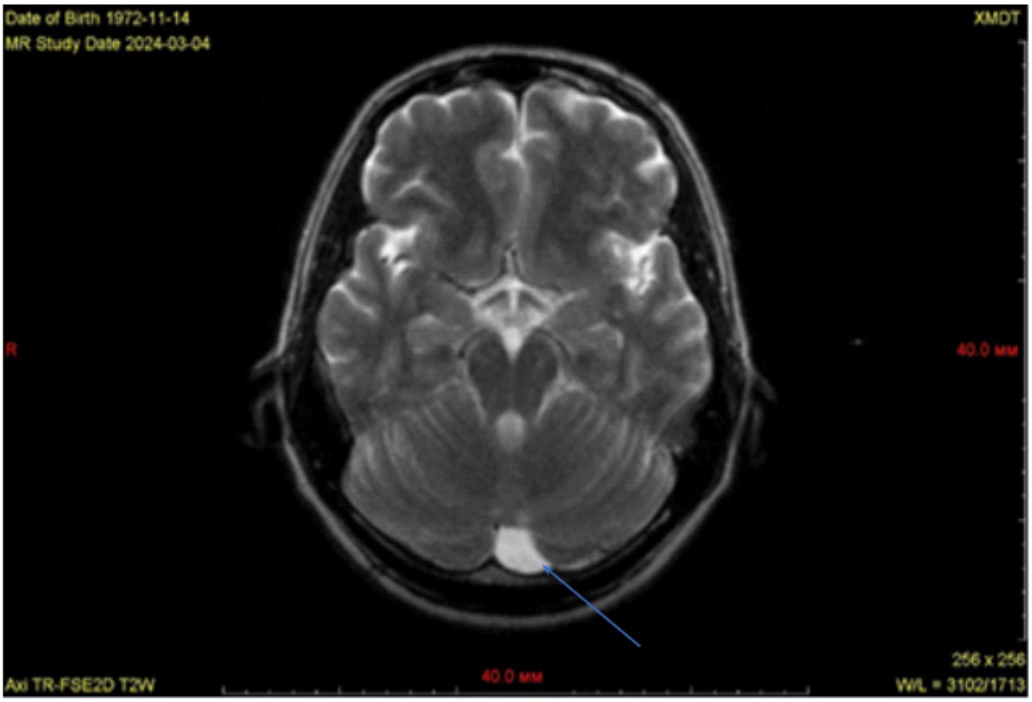

Fig. 2. Arachnoid retrocerebellar cysts in the military pilot in reserve S. This officer was discharged from military service due to reaching the maximum age limit. Brain MRI was not performed during previous VLEKs, the pilot was considered to be fit for flight duties

Table 1. Results of the professional psychological selection of the cadets of groups 1 (no brain alterations in MRI) and 2 (retrocerebellar cysts in MRI)

Category of professional psychological selection conducted at admission to the aviation school	Group 1, *n* = 307 (100%)	Group 2, *n* = 28 (100%)
1	8 (2%)	0 (0%)
2	268 (89%)	28 (100%)
3	31 (9%)	0 (0%)
4	0 (0%)	0 (0%)

Table 2. Average scores for the main academic disciplines and physical training in cadets of groups 1 (no brain alterations in MRI) and 2 (retrocerebellar cysts in MRI)

Academic performance	Group 1, no alterations based on MRI data (*n* ~307)	Group 2, alterations based on MRI and CT data (*n* ~28)
Average score for exams, 2nd semester, 1st year (2022–2023)	(*n* ~307) 4.2 ± 0.3	(*n* ~28) 4.1 ± 0.5
Average score for exams, 1st semester, 2nd year (2023–2024)	(*n* ~288) 251.0 ± 21.0	(*n* ~19) 248.0 ± 25.0
Average score for physical training, 2nd semester, 1st year (2022–2023)	(*n* ~307) 263.0 ± 22.0	(*n* ~28) 260.0 ± 26.0
Average score for physical training, 1st semester, 2nd year (2023–2024)	(*n* ~288) 4.3 ± 0.5	(*n* ~19) 4.2 ± 0.6

Table 3. Structure of reasons for expulsion from the aviation school in the first year among cadets of groups 1 (no brain alterations in MRI) and 2 (retrocerebellar cysts in MRI)

Reason for expulsion	Number of individuals expelled during their first year, no alterations in MRI Group 1 100% (*n* ~307)	Number of individuals expelled during their first year, alterations in MRI Group 2 100% (*n* ~28)
Poor academic performance	4 (1.3%)	1 (2.4%)
Indiscipline	4 (1.3%)	1 (2.4%)
VLEK results	0 (0%)	4 (10%)
Personal reasons	10 (3.3%)	3 (7.3%)

significant effect on the cerebrospinal fluid dynamics, and their clinical significance is poorly understood, which is in line with the data provided.

It should be noted that the presence of accidentally discovered cysts in two pilots in the reserve having a decent history of service, long flight experience on various types of aircraft and discharged from military service not for health reasons confirms the hypothesis about the negligible impact of retrocerebellar cysts found in pilots on their performance and professional longevity.

CONCLUSIONS

The findings undoubtedly require further research. However, the patterns revealed raise additional issues related to prediction of the impact of retrocerebellar cysts on the pilot's category of fitness determined during VLEK.

It is necessary to continue the research in order to gain the body of evidence and provide the possibility to predict the effects of retrocerebellar cyst in the brain on the cadets' performance and professional longevity.

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INTRACORONARY USE OF LEVOCARNITINE FOR CORONARY ARTERY STENT INSERTION IN HIGH-RISK PATIENTS

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The main causes of postoperative mortality associated with percutaneous coronary interventions involving the coronary artery stent insertion are perioperative myocardial infarction and acute heart failure due to inadequate protection of the myocardium against ischemia/reperfusion. The standard therapy includes beta blockers, anticoagulants, antiplatelet drugs. Two clinical cases of successful use of intravenous levocarnitine for cardioprotection in senile patients with acute forms of coronary heart disease with multivessel lesions are reported. The postoperative period went well, smooth dynamics of biomarker levels (troponin I, creatine phosphokinase, MB fraction of creatine phosphokinase) was observed, and ischemic ECG changes were relatively small. The expected results of the technique application include reduction of intraoperative and postoperative complications of ischemia/reperfusion and the increase in effectiveness of the stent insertion clinical outcomes in high-risk patients.

Keywords: coronary heart disease, acute coronary syndrome, myocardial infarction, ischemia/reperfusion, percutaneous coronary interventions, levocarnitine, intracoronary administration

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ИНТРАКОРОНАРНОЕ ПРИМЕНЕНИЕ ЛЕВОКАРНИТИНА ПРИ СТЕНТИРОВАНИИ КОРОНАРНЫХ АРТЕРИЙ У ПАЦИЕНТОВ ВЫСОКОГО РИСКА

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Основные причины послеоперационной летальности при выполнении чрескожных коронарных вмешательств со стентированием коронарных артерий — периоперационный инфаркт миокарда, аритмии и острая сердечная недостаточность вследствие неадекватной защиты миокарда от ишемии/реперфузии. Стандартная терапия включает бета-адреноблокаторы, антикоагулянты, дезагреганты. Описаны два клинических случая успешного внутривенного применения левокарнитина с целью кардиопротекции у пациентов старческого возраста с острыми формами ишемической болезни сердца при многососудистом поражении. Послеоперационный период протекал гладко, отмечалась сглаженность динамики биомаркеров (тропонин I, креатинфосфокиназа, МВ-фракция креатинфосфокиназы), ишемические сдвиги ЭКГ были мало выражены. Ожидаемые результаты применения методики — снижение интраоперационных и послеоперационных осложнений ишемии/реперфузии и повышение эффективности клинических результатов стентирования у пациентов высокого риска.

Ключевые слова: ишемическая болезнь сердца, острый коронарный синдром, острый инфаркт миокарда, ишемия/реперфузия, чрескожные коронарные вмешательства, левокарнитин, внутрикоронарное введение

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Today, percutaneous coronary interventions (PCI) are performed annually in at least 5,000,000 patients with coronary heart disease (CHD) all over the world [1], among them more than 200,000 are performed annually in Russia [2]. The major causes of postoperative mortality associated with such interventions include perioperative myocardial infarction (MI), arrhythmia,

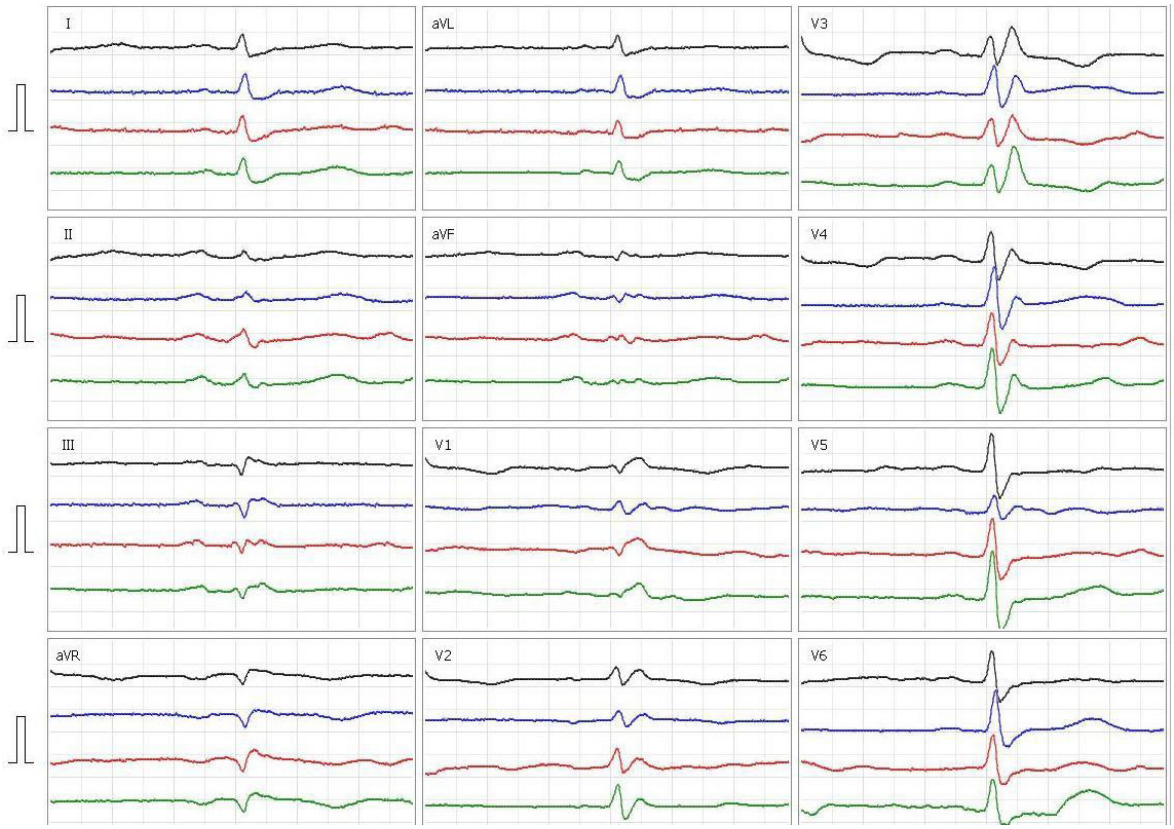

Fig. 1. Dynamic changes in ECG of patient H. aged 76: ECG recorded on 20.09.2023 (before LC administration) is highlighted in *green*; ECG recorded on 21.09.2023 is highlighted in *red*, ECG recorded on 25.09.2023 is highlighted in *blue*; ECG recorded on 26.09.2023 (after LC administration) is highlighted in *black*

and acute heart failure due to inadequate protection of the myocardium against ischemia/reperfusion under conditions of ballooning and stent insertion into the affected coronary arteries.

The recent clinical guidelines of the European Society of Cardiology (ESC) on treatment of acute coronary syndrome (ACS) issued in 2023 [3] specify that various strategies to protect the myocardium during PCI are studied in preclinical and clinical trials: coronary postconditioning, remote ischemic conditioning, early intravenous administration of metoprolol, glycoprotein IIb/IIIa inhibitors, drugs designed to preserve integrity of mitochondria, nitric oxide production. Adenosine, glucose modulators, hypothermia and other techniques are considered as cardioprotectors. Thus, additional cardioprotection means are nowadays in high demand, while the strategies to reduce ischemia/reperfusion damage generally remain an unfulfilled clinical need.

We believe that levocarnitine (L-carnitine, γ-trimethylamino-β-hydroxybutyrate, LC), the natural endogenous component of mammalian tissues, is a very promising agent in terms of myocardial protection. The LC antioxidant effect in ischemia/reperfusion can be associated with the free fatty acid metabolism optimization and attenuation of the inhibiting effect of reactive oxygen species on the aerobic metabolism [4]. It has been confirmed that the levels of LC drop sharply in the ischemic myocardium of individuals with acute MI, cardiomyopathy, and heart failure of various origin [5, 6].

Practically the first Russian study that revealed beneficial effects of LC on the myocardium in acute MI [7] was represented by the study focused on assessing the protective effects of intravenous LC in ACS, where the left ventricular ejection fraction (LVEF) reduction to less than 40% was the inclusion criterion [8].

The LC beneficial effects on the course of ACS were also confirmed by other authors. Thus, intravenous LC therapy reduced the corrected QT dispersion, the NO bioavailability was enhanced [9, 10]. Beneficial effects of using LC in patients with ACS were reported within the early period, along with good tolerability of the drug [11]. In individuals with acute MI, there was a significant decrease in the average peak levels of myocardial necrosis markers in blood (CPK-MB and troponin) [12]. Intravenous administration of LC turned out to be effective in myocardial ischemia/reperfusion injury, ventricular dysfunction, heart arrhythmia, and toxic myocardial damage. The LC beneficial effects were reported in infants, adolescents, young adults, adults, and elderly patients with acute and chronic heart failure (CHF) [13–15].

In the past, trimetazidine [16] and phosphocreatine (Neoton) [17] were used for adjuvant cardioprotection during percutaneous interventions, which was not very successful and did not become a common myocardial protection method.

Clinical cases

We have earlier provided the results of the first clinical use of intracoronary levocarnitine in patients with acute MI [18]. Here we provide another two cases of using the method during emergency PCI in senile patients.

Observation 1

Patient H. aged 76 underwent inpatient treatment in the cardiology unit of the Saint Petersburg Clinical Hospital of the Russian Academy of Sciences between 20.09.2023 and 26.09.2023. According to the medical history, H. had been suffering from moderate hypertension for a couple of years. He insisted he had no acute MI or acute cerebrovascular accident (CVA). In 2018, sigmoid cancer was detected, surgery was performed, and the patient received the course of chemotherapy.

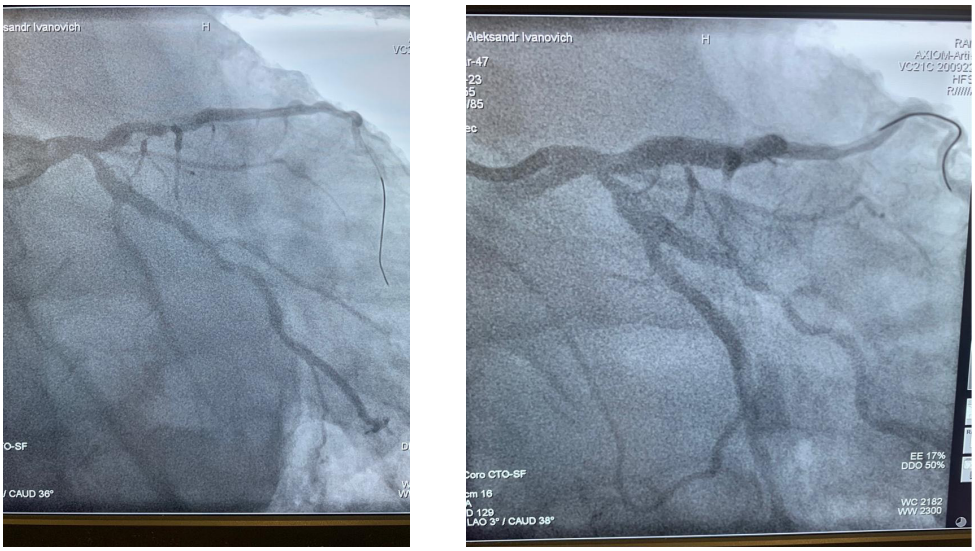

Fig. 2. Angiography of the affected coronary artery of patient H. performed before (*on the left*) and after (*on the right*) the stent insertion

In 2019, prostate cancer was revealed, due to which the patient received endocrine therapy every 3 months. Since 2017 he was followed up by angiosurgeons due to infrarenal aortic aneurysm. Previously, stent insertion into the anterior interventricular artery was performed. Starting from the night of 20.09.2023, the recurrent attacks of anginal pain emerged, due to which the patient used nitrospray three times with a positive effect. In the morning of 20.09.2023, the emergency hospitalization took place, followed by diagnostic coronary angiography with immediate stent insertion into the RCA (three BioMatrix Flex stents; Singapore). To reduce the extent of ischemic damage, intracoronary administration of the 1000 mg LC solution (Elcar) was performed. The patient received Elcar in a dose of 500 mg per day (No. 7) between 20.09.2023 and 26.09.2023. According to the echocardiography (ECHO) data, LVEF was 53% on 20.09.2023 and 56% on 22.09.2023. The dynamic changes in echocardiography (ECG) data are provided in Fig. 1, angiography data are presented in Fig. 2, and the dynamic changes in biomarkers of myocardial ischemia/necrosis are provided in Table 1.

Observation 2

Female patient L. aged 78 underwent inpatient treatment at the Saint Petersburg Clinical Hospital of the Russian Academy of Sciences between 25.09.2023 and 30.09.2023. Medical history: essential hypertension for many years, type 2 diabetes mellitus diagnosed in 2006. The CHD onset occurred in 2017, stent insertion into the circumflex branch (CB) of the left coronary artery (LCA) (two stents) was performed. Aggravation of symptoms started from March 2023: shortness of breath and chest pain during exercise emerged. Standard therapy was supplemented by prescription of the course of meldonium in outpatient settings, diuretic therapy was added. However, progressive worsening was observed. Echocardiography performed in August 2023 revealed LVEF reduction to 30%. On the night of 25.09.2023 the patient noted a burning sensation around her heart at rest. She was diagnosed with ACS without ST segment elevation on ECG and underwent emergency diagnostic coronary angiography (LMCA stenosis 90%, CB outflow subocclusion) followed by angioplasty with stent insertion into the CB. To reduce the extent of ischemic damage, intracoronary administration of the 1000 mg LC (Elcar) solution was performed.

The patient received intravenous infusions of Elcar in a dose of 500 mg per day (No. 5) between 26.09.2023 and 30.09.2023. According to the echocardiography (ECHO) data, LVEF was 36% on 25.09.2023 and 39% on 27.09.2023. The dynamic changes in ECG are provided in Fig. 3, angiography is presented in Fig. 4.

The dynamic changes in biomarkers of myocardial ischemia/necrosis in patient L. are provided in Table 2.

Clinical case discussion

The literature data on the successful use of intravenous LC medication aimed to ensure cardioprotection in patients with CHD substantiate the research focused on assessing the intracoronary drug administration route, since this substance exerts pronounced protective effects confirmed both *in vivo* and *in vitro* (antioxidant, anti-ischemic and metabolic, i.e. related to the cell energy supply). It is PCI in high-risk patients (elderly and senile patients with multivessel coronary lesions), during which it would be possible to assess the efficacy, response time, safety, and probable dose dependence of such protection.

The fact that the tissue levels of LC decrease with age in individuals with chronic and especially acute CHD is one more

Table 1. Dynamic changes in the levels of myocardial ischemia/necrosis biomarkers in patient H.

	Reference values	20.09.2023	21.09.2023	22.09.2023	25.09.2023
High-sensitivity troponin I (hsTnI)	0–39,2 pg/mL	5	2371	1684	555
ALT	10–40 U/L	16	19	18	19
AST	10–40 U/L	17	25	18	18
CPK	0–190 U/L	81	228	90	
CPK-MB	0–24 U/L	9	20	8	10

Note: ALT — alanine aminotransferase, AST — aspartate aminotransferase, CPK — creatine phosphokinase, CPK-MB — creatine phosphokinase-MB.

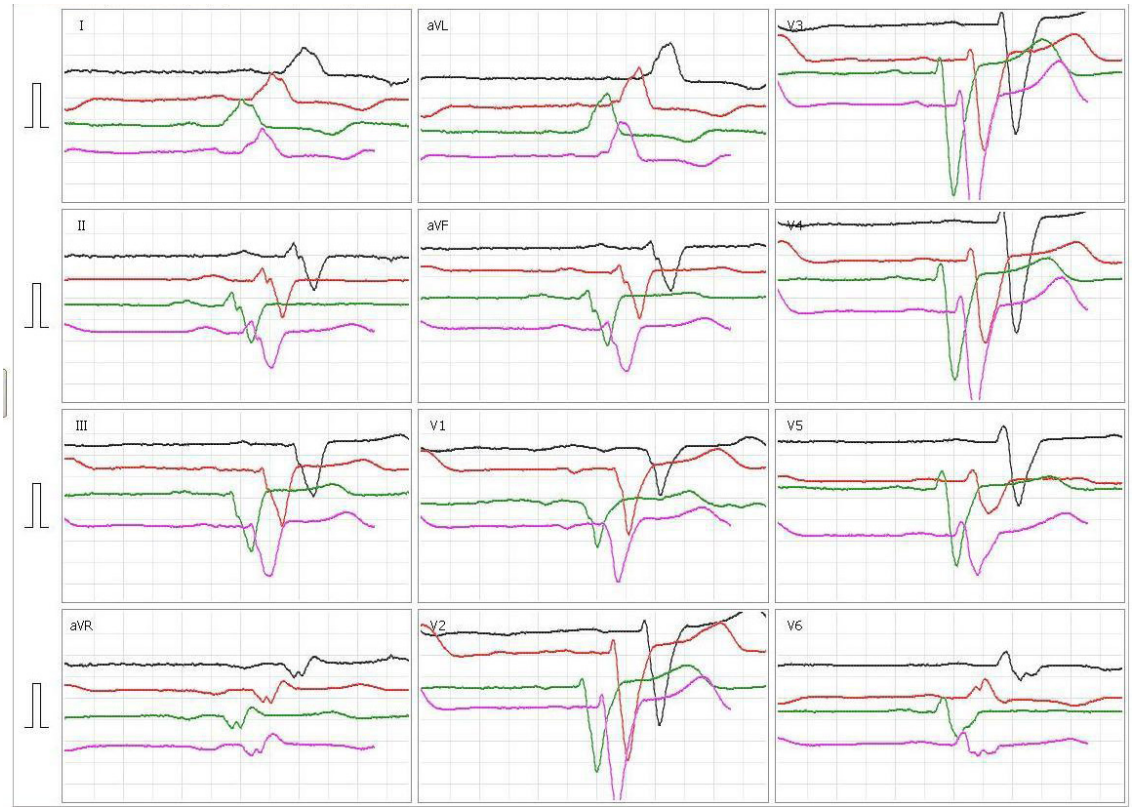

Fig. 3. Dynamic changes in ECG of patient L. aged 76: ECG recorded on 25.09.2023 at 10:32, before LC (Elcar) administration, is highlighted in *black*; ECG recorded on 25.09.2023 at 18:52, after LC (Elcar) administration, is highlighted in *red*; ECG recorded on 26.09.2023 is highlighted in *green*; ECG recorded on 29.09.2023 is highlighted in *pink*

reason to study the LC efficacy in the high-risk elderly patients with CHD [19]. It is also important that LC used as part of the combination drug therapy demonstrates an improved safety profile in patients with comorbidities, exerting no effect on P450 cytochrome [20, 21].

Given the data of the large-scale meta-analysis of 13 randomized controlled trials (RCTs) focused on the secondary prevention of cardiovascular disorders (comparison of LC and placebo), we can expect the decrease in the rate of ventricular arrhythmias and angina attacks in patients having a history of MI [22]. It seems quite likely that manifestations of heart failure will decrease when using the described technique, since, according to meta-analysis of 17 RCTs focused on using LC in patients with CHF [23], the increase in LVEF, optimization of the LV end-diastolic and end-systolic volume, significant decrease in blood levels of brain natriuretic peptide (BNP) and N-terminal pro b-type natriuretic peptide (NT-proBNP) after administration of the drug have been reported [24].

Apparently, the postoperative period went relatively well in both reported cases; smooth dynamics of biomarker levels (troponin I, creatine phosphokinase, MB fraction of creatine phosphokinase, transaminases) and the systolic function stability were observed; the ischemic ECG changes were relatively small.

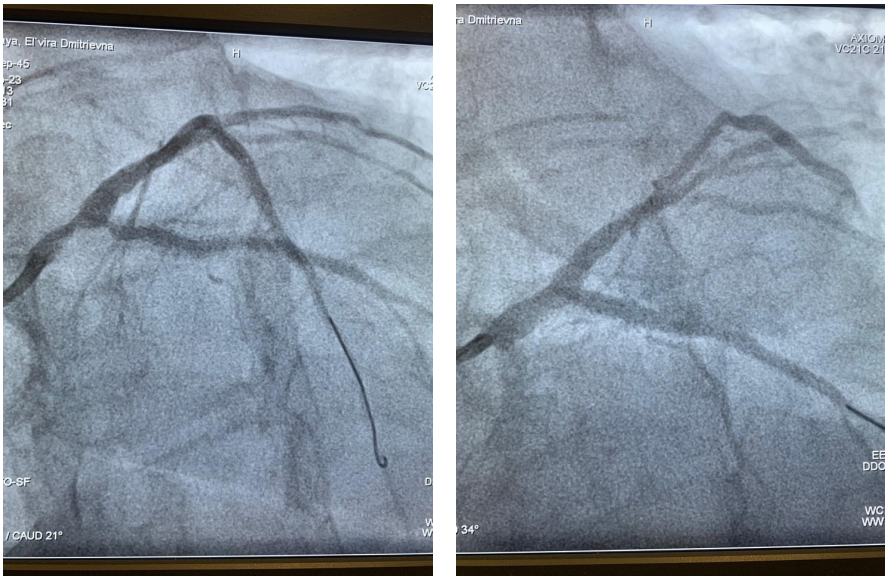

Fig. 4. Angiography of the affected coronary artery of patient L. performed before (*on the left*) and after (*on the right*) the stent insertion

Table 2. Dynamic changes in the levels of myocardial ischemia/necrosis biomarkers in patient L.

	Reference values	25.09.2023	27.09.2023	28.09.2023
High-sensitivity troponin I (hsTnI)	0–39,2 pg/mL	12.1	132.2	73.9
ALT	10–40 U/L	20	39	15
AST	10–40 U/L	18	28	17
CPK	0–190 U/L			83
CPK-MB	0–24 U/L	7	9	8

Note: ALT — alanine aminotransferase, AST — aspartate aminotransferase, CPK — creatine phosphokinase, CPK-MB — creatine phosphokinase-MB.

CONCLUSION

Thus, the proposed technique involving intracoronary LC administration during PCI in high-risk patients can become effective for protection of myocardium against the ischemia/reperfusion complications, which will improve the outcomes of revascularization and reduce the rate of complications. Strong conclusions can be drawn only after accumulation and analysis of sufficient data within the framework of the expected future research due to the paucity of available observations.

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CLINICAL FEATURES OF PROTRACTED INTESTINAL INFECTION ASSOCIATED WITH *KLEBSIELLA PNEUMONIAE* IN AN INFANT

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The development of complex criteria for the diagnosis, differential diagnosis, and optimization of treatment of infectious diarrhea associated with opportunistic *Enterobacteriaceae* is a pressing issue of pediatric research and practice. The paper reports a clinical case of protracted intestinal infection associated with *Klebsiella pneumoniae* in the form of moderate hemorrhagic enterocolitis in an infant, which is explained by the decrease in specific resistance due to unfavorable maternal obstetric and gynecological history, perinatal CNS injury, iron deficiency anemia, protein-energy malnutrition. The disease relapse associated with secondary norovirus infection was reported after the first hospitalization. Three courses of intestinal antiseptics and probiotics were required to achieve a beneficial treatment outcome, although usually in such a situation one course of such drugs is enough. The recovery process was accompanied by the nutritional status improvement, hemorrhagic colitis relief, normalization of gut microbiota.

Keywords: intestinal infections, infants, opportunistic enterobacteria, diagnosis, treatment

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Compliance with the ethical standards: the informed consent to publication of case report was obtained from the patient's parents.

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КЛИНИЧЕСКИЕ ОСОБЕННОСТИ ЗАТЯЖНОГО ТЕЧЕНИЯ КИШЕЧНОЙ ИНФЕКЦИИ, АССОЦИИРОВАННОЙ С *KLEBSIELLA PNEUMONIAE*, У РЕБЕНКА ГРУДНОГО ВОЗРАСТА

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Разработка комплексных критериев диагностики, дифференциальной диагностики и оптимизации лечения инфекционных диарей, ассоциированных с условно-патогенными энтеробактериями, — одна из важных задач научной и практической педиатрии. Представлен клинический случай затяжного течения кишечной инфекции, ассоциированной с *Klebsiella pneumoniae*, протекавшей в виде геморрагического энтероколита в среднетяжелой форме, у ребенка грудного возраста, что объяснялось снижением неспецифической резистентности по причине неблагоприятного акушерско-гинекологического анамнеза матери, перинатального поражения ЦНС, железодефицитной анемии, белково-энергетической недостаточности. После первой госпитализации на фоне присоединения норовирусной инфекции отмечали рецидив заболевания. Для достижения положительного эффекта лечения потребовалось проведение трех курсов кишечных антисептиков и пробиотиков, хотя обычно в подобной ситуации достаточно одного курса данных препаратов. Процесс выздоровления сопровождался улучшением состояния питания, купированием гемоколита, нормализацией микробиоты кишечника.

Ключевые слова: кишечные инфекции, дети раннего возраста, условно-патогенные энтеробактерии, диагностика, лечение

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Соблюдение этических стандартов: от родителей пациента было получено добровольное информированное согласие на публикацию клинического случая.

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The incidence of acute intestinal infections (AII) is a pressing issue. According to the World Health Organization (WHO), more than a billion AII cases are reported annually, among them 20 million are severe and 1/2 of fatal cases occur in children under the age of 5 years [1]. Furthermore, the percentage of AII cases associated with opportunistic enterobacteria is 12.8% [2].

Today, *Klebsiella pneumoniae* is the leading opportunistic pathogen causing AII [3, 4]. The majority of patients is represented by infants with underdeveloped gut microbiota and immature immune system showing signs of chronic nutritional disorders and anemia that adversely affect the nonspecific resistance [5–7] and contribute to the protracted course of AII followed by the development of gastrointestinal disorder [8–11].

The development of complex criteria for the diagnosis, differential diagnosis, and optimization of treatment tactics for infectious diarrhea, including that caused by opportunistic pathogens, aimed to improve outcomes in children is a pressing issue of pediatric research and practice [3, 12–14].

The study was aimed to assess clinical features of protracted AII associated with *K. pneumoniae* in an infant in order to reveal the diagnosis and treatment problems.

Clinical case

The clinical case of AII associated with *K. pneumoniae* in an infant admitted three times to the department of intestinal infections of the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia is reported. When making a diagnosis, we assessed medical history, clinical symptoms, results of objective examination and laboratory tests (complete blood count, blood biochemistry test, urinalysis, coprogram), instrumental screening data (ECG, ECHO; abdominal, renal, bladder ultrasound; neurosonography, EEG). The AII etiology was verified by fluorescent polymerase chain reaction (PCR) using the AmpliSens® OKI-screen-FL kit (FBIS CRIE; Russia) for qualitative detection and differentiation of bacterial DNA of *Shigella spp*. and enteroinvasive *E. coli* (EIEC), *Salmonella spp*., and thermophilic *Campylobacter spp*. in fecal samples, as well as DNA of *Adenovirus* F and RNA of *Rotavirus* A, genotype 2 N*orovirus*, astroviruses; bacteriological testing of feces for bacteria of the typhoid-paratyphoid-dysentery group, *Campilobacter spp*., opportunistic *Enterobacteriaceae*; enzyme-linked fluorescence assay involving determination of *C. difficile* A and B toxins in the feces; serological testing aimed to reveal antibodies against *S. sonnei*, *S. flexneri*, *Salmonella spp*., *Y. enterocolitica* O3, *Y. enterocolitica* O9. Gut dysbiosis was detected based on the abundance of atypical *E. coli* in the feces (lg CFU/mL).

The boy M. aged 2 months 17 days arrived by ambulance on 13.03.2023 complaining of diarrhea with blood and mucus.

Medical history. Had been sick for 2 weeks; amid the onset of profuse regurgitation and intestinal colic, the appetite loss, weight loss, large amounts of mucus in watery bowel movements (3–4 a day) were observed. Regurgitation became more frequent, and blood streaks in stool (3–4 times a day) emerged in the last three days (Table 1).

Life history. The child was born to a primagravida having gestosis and threatened miscarriage during pregnancy. Delivery at term. Birth weight 3060 g, body length 50 cm. Normal neonatal period. The child was breastfed. Weight gain in the first 2 months was 900 g per month. Vaccinated against tuberculosis and hepatitis B in the maternity hospital. No hereditary burden.

The infant's condition at admission was of moderate severity. Body temperature 36.0 °C. Body length 60 cm (4 points). Body weight for stature 5270 g (2 points). Clear consciousness. Skin and mucous membranes were pale pink and clean. Nutritional status is moderately reduced (weight deficit exceeding 10%). No skin turgor decrease. The degree of dehydration according to the WHO clinical scale was mild. No hyperemia

Table 1. Features of the course of AII associated with *K. pneumoniae* in an infant

Signs of the disorder	Follow-up periods					
	hospital stay		hospital stay		hospital stay	
	13.03	21.03	03.04	13.04	18.05	24.05
Age	2 months 17 days		3 months 10 days		4 months 24 days	
Body lengh, cm (points)	60.0 (4)	60.0 (4)	60.0 (4)	60 (3)	60 (2)	60 (2)
Body weight for stature, kg (points)	5.270 (2)	5.300 (3)	5.720 (4)	5.920 (4)	5.740 (4)	5.850 (4)
Body temperature, °C	36	36.7	36.3	36.5	36.5	36.7
Fatigue		–		–		–
Loss of appetite	+ 2 weeks	–	+ c 31.03	–	+ starting from 17.04.23	–
Decrease in weight gain	+ 2 weeks	–	±	+	+	–
Profuse spitting up, rare vomiting	+	–	+	–	–	–
Intestinal colic		–		–		–
Flatulence		–		–		–
Watery stool	2 weeks, 3–4 times a day	mushy	+ starting from 01.04.23	mushy	dilute, 1–2 times a day	–
Large amounts of mucus in watery stool	2 weeks, 3–4 times a day	–	+ starting from 01.04.23	–	±	–
Traces of blood in liquid stool	3 days, 3–4 times a day	–	+ starting from 01.04.23	–	–	–
Diuresis	preserved	normal	preserved	normal	preserved	normal
Primary clinical diagnosis	A04.8 — Other specified bacterial intestinal infections. Acute gastroenterocolitis associated with *K. pneumoniae* of moderate severity		A08.1 — Acute gastroenteropathy due to norovirus. Acute gastroenteritis of moderate severity		A09 — Other and unspecified enterocolitis, mild form	
Secondary diagnosis	D50.9 — Iron deficiency anemia, mild form. E44 — Protein-energy malnutrition of moderate and mild degree		A04.8 — Other specified bacterial intestinal infections. Acute enteritis, hemorrhagic colitis associated with *K. pneumoniae*, moderate form, protracted course. D50.9 — Iron deficiency anemia, mild form		D50.9 — Iron deficiency anemia, mild form	
Complications of primary condition	E87 — Other disorders of fluid, electrolyte and acid-base balance, exicosis of I degree		E87 — Other disorders of fluid, electrolyte and acid-base balance, exicosis of I degree			

Table 2. Data of laboratory testing of an infant having AII associated with *K. pneumoniae* acquired during treatment in hospital settings

Parameters	Normal	Testing dates				
		hospital stay		hospital stay		hospital stay
		13.03.23	20.03.23	03.04.23	11.04.23	18.05.23
Complete blood count						
White blood cells, 10^9/L	6.0–17.5	5.6	6.3	11.12	12.25	8.5
Red blood cells, 10^{12}/L	3.6–4.9	3.2	3.4	3.26	3.25	3.85
Hemoglobin, g/L	110–135	96	100	89	90	102
Hematocrit, %	33.0–47.5	27.6	28.1	26.6	26.7	29.9
Mean corpuscular volume, fl	70.0–84.0	86.1	84.1	81.7	82.1	77.8
Platelets, 10^9/L	180–400	398	635	483	470	438
Thrombocrit, %	0.10–0.40	0.34	0.55	0.44	0.43	0.4
Neutrophils, %	15.5–49.0	30.4	29.3	22.8	27	26.2
Lymphocytes, %	38.0–72.0	61	75	67.7	61.4	64.6
Monocytes, %	2.0–12.0	1	1	7	7.3	6.2
Eosinophils, %	0.0–6.0	1	5	2.5	4.3	3
Eosinophils, 10^9/L	0.10–1.00	0.06	0.15	0.28	0.53	0.25
ESR, mm/h	2–17	25	10	18	7	3
Blood biochemistry test						
ALT, U/L	0.00–55.0	28		41.6		35
Urea, mmol/L	2.78–8. 07	3.66	2.35	1.67		1.87
Total bilirubin, μmol/L	0.00–21.00					3
Creatinine PAP, μmol/L	15.0–37.0	41	22	35		24
Glucose, mmol/L	3.5–5.8	5.1		5.2		5.2
Amylase, U/L	28.0–100.0	9		12		15
C-reactive protein	0.0–5.0			1.4		
Iron, μmol/L	9.5–30.0			8		8.3
Blood electrolytes						
Potassium, mmol/L	3.7–5.7	6	5.7	5.6		4.7
Sodium, mmol/L	130–145	135	134	135		136
Calcium, mmol/L	1.00–1.29	1.28		1.27		1.27
Coprogram						
Fecal occult blood		+	–	–	–	–
Color		greenish yellow	greenish yellow	yellowish brown	yellowish brown	greenish yellow
Texture		mushy	mushy	loose	mushy	mushy
pH		6	6	6	7	6
White blood cells in mucus		18–20	8–10	–	–	3–5
Red blood cells		–	–	–	–	–
Mucus		+++	+++	–	–	+
Bacteriological testing of feces						
Testing dates		13.03.23	20.03.23	03.04.23	11.04.23	18.05.23
Klebsiella pneumoniae, CFU/mL		10^6	10^5	10^6		10^4
E. coli, nontypeable, non-lactose fermenting, CFU/mL		10^6		10^6		
E. coli, nontypeable, lactose fermenting, CFU/mL			10^3			10^3

of the oropharyngeal mucosa. No peripheral lymph node enlargement, painless lymph nodes. Normal musculoskeletal system. Pulse rate 138 bpm. BP 90/64 mmHg. No expansion of cardiac borders, tone was clear, rhythmic. Respiratory rate 26 breaths per minute. Puerile respiration. Percussion sound is pulmonary. The abdomen was soft and painless. The liver was palpated at 1–1.5 cm below the costal margin; no enlargement of the spleen. Yellow-green liquid stool with mucus and traces of blood (examined). Preserved diuresis.

The complete blood count test revealed decreased counts of white blood cells, red blood cells, decreased levels of hemoglobin, hematocrit, decreased counts of monocytes, eosinophils, increased erythrocyte sedimentation rate (ESR) (Table 2), which were indicative of inflammation and grade 1 anemia. Blood biochemistry test revealed elevated creatinine, potassium levels, along with the decreased amylase level, which were considered to be manifestations of acute kidney injury and decreased secretory function of the pancreas resulting

Table 3. Features of treatment of an infant having All associated with *K. pneumonia*

Therapy	hospital stay 13.03–21.03	outpatient clinic	hospital stay 03.04–13.04	outpatient clinic	hospital stay 18.05–25.05	outpatient clinic
Feeding	breastfeeding	breastfeeding + lactose-free formula supplementation	breastfeeding + lactose-free formula supplementation	breastfeeding + lactose-free formula supplementation	breastfeeding + weaning foods on water	breastfeeding + weaning foods
Oral rehydration	rehydration solution for children	–	rehydration solution for children	–	rehydration solution for children	–
Sorbents	–	–	–	–	hydrolytic lignin	–
Probiotics	+	+	+	+	+	+
Intestinal antiseptics	nifuroxazide 5 days	nifuroxazide 5 days	nifuratel 4 days	nifuratel 6 days	–	–
Antifoam agents	simethicone 6 days	simethicone when required	simethicone 9 days	–	–	–
Prokinetic agentы	–	–	domperidone 6 days	–	–	–
Digestive enzymes	dietary supplement being the source of lactase	–	–	–	–	–
Iron supplements	–	iron(III)-hydroxide polymaltose complex	iron(III)-hydroxide polymaltose complex	iron(III)-hydroxide polymaltose complex	iron(III)-hydroxide polymaltose complex	iron(III)-hydroxide polymaltose complex

from the intoxication and dehydration syndromes. Occult blood was revealed in the feces by the Gregersen fecal occult blood test; white blood cells up to 20 per field of view, large amounts of mucus, and the lack of red blood cells were revealed by microscopy. Bacteriological testing revealed growth of *K. pneumoniae* in the diagnostically significant titer of 10^6 CFU/mL (susceptibility to amoxicillin, ceftriaxone, gentamicin, nalidixic acid, nitrofurantoin, trimethoprim; resistance to the *Klebsiella K. pneumoniae* polyvalent bacteriophage were reported), which provided the basis for the following etiological diagnosis: A04.8 — Other specified bacterial intestinal infections. Acute gastroenterocolitis associated with *K. pneumoniae* of moderate severity (Table 1). The growth of nontypeable non-lactose fermenting *E. coli* in a high titer, 10^6 CFU/mL, was observed that was indirect evidence of gut dysbiosis.

According to the instrumental diagnostic screening data (ECG, ECHO; abdominal, renal, bladder ultrasound), the following was revealed: incomplete right bundle branch block; hemodynamically insignificant patent foramen ovale, left ventricular accessory chord; gallbladder deformity, moderate enlargement of the liver, fluid filled bowel loops, bowel wall thickening up to 2 mm.

Treatment included diet therapy (breastfeeding reduced per actual body weight), oral rehydration, intestinal antiseptics (nifuroxazide, 100 mg 3 times a day), probiotic, enzyme products (dietary supplement being the source of lactase), symptomatic therapy (simethicone) (Table 3). Treatment resulted in feeling better, relief of vomiting and profuse spitting up regurgitation, resolution of flatulence, stool back to normal; however, the weight gain was insufficient. Red blood counts improved; white blood cell counts and ESR were back to normal; the increase in platelet counts, thrombocrit, relative lymphocyte counts was reported. The creatinine, potassium levels were back to normal; however, there was a significant decrease in urea levels being an indirect evidence of inhibition of synthetic function of the liver under the influence of infection [8].

The patient was discharged after 7 days due to clinical improvement, his body weight was 5300 g (+ 30 g; 3 points); the follow-up bacteriological testing of the feces revealed the decrease in the *K. pneumoniae* titer to 10^5 CFU/mL, the lack of nontypeable non-lactose fermenting *E. coli*, and the emergence of nontypeable lactose fermenting *E. coli* in a titer of 10^3 CFU/mL. It was recommended to continue treatment in outpatient settings.

The second hospitalization took place 13 days later. The child was admitted due to referral from local pediatrician complaining of bloating, intestinal colic, spitting up regurgitation, vomiting, recurrent breast refusal, watery bowel movements (3–4 a day) since 30.03.2023, fatigue since 31.03.2023, increase in body temperature to 37.5 °C since 02.04.2023. Starting from 24.03.2023, blood streaks in stool were noted; the child had been receiving nifuroxazide for 5 days, 100 mg 3 times a day, showing improvement. Blood streaks in stool were noted again starting from 01.04 (Table 1).

The condition at admission was of moderate severity. Body temperature 36.4 °C. Body length 60 cm (4 points). Body weight for stature 5720 г (4 points) (Table 1). Clear consciousness. Skin and mucous membranes were pale pink and clear. Nutritional status is satisfactory. No skin turgor decrease. The degree of dehydration according to the WHO clinical scale was mild. No hyperemia of the oropharyngeal mucosa. No peripheral lymph node enlargement, painless lymph nodes. No apparent abnormality of the musculoskeletal system. Pulse rate 148 bpm. BP 90/57 mmHg. No expansion of cardiac borders, tone was clear, rhythmic. Respiratory rate 34 breaths per minute. Puerile respiration. Percussion sound is pulmonary. The abdomen was soft and painless. The liver was palpated at 1–1.5 cm below the costal margin; the spleen was not palpable. Yellowish brown loose stool with no abnormal foreign matter (examined). Preserved diuresis.

Laboratory testing revealed grade 1 anemia, moderate thrombocytosis, increased ESR, decreased serum levels of iron, urea, amylase. Normal coprogram. A norovirus antigen was detected in the feces; growth of *K. pneumoniae* in a high titer of 10^6 CFU/mL (with the antibiotic susceptibility and bacteriophage resistance similar to that revealed during the first hospitalization) was observed; growth of nontypeable non-lactose fermenting *E. coli* in a titer of 10^6 CFU/mL was reported, which, in aggregate, made it possible to establish the diagnosis of acute norovirus gastroenteritis of moderate severity

combined with protracted AII (enteritis, hemorrhagic colitis) associated with *K. pneumoniae*, which took place against the background of gut dysbiosis (Table 1). Abnormal foreign matter typical for colitis was visually detected in the feces starting from days 6–7 of hospital stay.

Based on the neurological assessment and neurosonography data it was reported that the child had perinatal CNS injury, moderate hypotonia, and was through early recovery period.

Treatment included diet therapy (breastfeeding with lactose-free formula supplementation), oral rehydration, probiotic, intestinal antiseptics (nifuratel in a dose of 10 mg/kg 3 times a day), symptomatic therapy (simethicone, domperidone), iron supplement (iron (III) — hydroxide polymaltose complex) (Table 3).

The patient was discharged after 10 days due to clinical improvement. It was recommended to continue treatment in outpatient settings.

The third hospitalization took place after 37 days. The infant's parents contacted the clinic without any referral from the local physician. Loss of appetite, sometimes watery stool with mucus (1–2 times a day), bloating, restlessness were observed starting from 17.05.2023; dilute stool with small amounts of mucus (up to 3–4 times a day), fatigue were reported starting from 18.05.2023.

The condition at admission was of moderate severity. Body temperature 36.5 °C. Body length 60 cm (2 points). Body weight for stature 5740 g (4 points) (Table 1). Clear consciousness. Skin and mucous membranes were pale pink and clean. Nutritional status is satisfactory. No dehydration according to the WHO scale. No hyperemia of the oropharyngeal mucosa. No peripheral lymph node enlargement, painless lymph nodes. No evident musculoskeletal system abnormality. Pulse rate 142 bpm. BP 80/50 mmHg. No expansion of cardiac borders, tone was clear, rhythmic. Respiratory rate 42 breaths per minute. Puerile respiration. Percussion sound is pulmonary. Soft and painless abdomen. The liver was palpated at 1–1.5 cm below the costal margin; no enlargement of the spleen. Greenish yellow and mushy stool, large amounts of mucus (examined). Preserved diuresis.

Testing revealed the signs of grade 1 anemia, moderate thrombocytosis, slight upward trend in blood levels of iron, preserved low serum levels of urea and amylase. Normal coprogram. A significant decrease in the *K. pneumoniae* titer (to 10^4 CFU/mL) together with the presence of nontypeable lactose fermenting E. coli in a titer of 10^3 CFU/mL in the feces was observed, which suggested the child's recovery from AII associated with *K. pneumoniae*, gut microbiota composition improvement. Primary clinical diagnosis at discharge: A09 — Other and unspecified enterocolitis, mild form (Table 2).

The treatment applied during the last hospitalization included diet therapy (breastfeeding, weaning foods on water), oral rehydration, enterosorbent (hydrolytic lignin), probiotic, iron supplement (iron (III) — hydroxide polymaltose complex) (Table 3).

The child was discharged after 6 days due to general health improvement and stool back to normal.

Clinical case discussion

This clinical case demonstrates typical features of protracted AII associated with *K. pneumoniae* in the form of gastroenterocolitis (hemorrhagic colitis). The disease onset was associated with the decrease in nonspecific resistance resulting from unfavorable maternal obstetric and gynecological history, perinatal CNS injury with decreased muscle tone, iron deficiency anemia, protein-energy malnutrition, which was largely compliant with the data reported by other researchers [3, 4, 6]. The gut dysbiosis accompanying perinatal abnormalities and deficiencies in infants also contributed to the protracted course of AII associated with the opportunistic representative of *Enterobacteriaceae* [3, 5] and showed remarkable persistence, despite the repeated courses of probiotic therapy. It is clear that the hemorrhagic colitis relapse during the second hospitalization was caused by activation of opportunistic gut microbiota associated with norovirus infection layering. Recovery was accompanied by the nutritional status improvement, hemorrhagic colitis relief, switch from mixed feeding to breastfeeding due to restoration of lactation in the mother, however, the child's physical development was still disharmonious, which was explained by persistence of iron deficiency anemia and metabolic disorder. It seems that the child's third hospitalization resulted from the functional gastrointestinal disorder, not from the new episode of AII of unknown etiology.

CONCLUSION

In modern conditions, when there are no clinical guidelines on management of infants with intestinal infection associated with opportunistic Enterobacteriaceae, practitioners rely on the experts showing the possibility of etiological and differential diagnosis of this disorder. However, the issue of *K. pneumoniae* significance in community-acquired pediatric AII is still not completely resolved.

Treatment of patients traditionally includes diet therapy, rehydration, enterosorption, probiotics. Intestinal antiseptics are prescribed according to indications, one of which is hemorrhagic enterocolitis; however, the etiotropic treatment efficacy is not always sufficient. It seems that this problem can be solved through testing and implementation of personalized treatment approaches based on autoprobiotics and/or *Klebsiella bacteriophages* being an alternative to antibacterial agents used against *K. pneumoniae*.

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