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# ACTA BIOMEDICA SCIENTIFICA

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# DEPUTY EDITOR-IN-CHIEF'S PREFACE

## DEPUTY EDITOR-IN-CHIEF'S PREFACE TO ISSUE 1, 2024

**Lyubov V. Rychkova**

**Dr. Sc. (Med.), Professor, Corresponding Member the of RAS**

In the first issue of the journal for 2024, experimental research takes a leading position. Its high percentage in the total number of articles indicates the progress in scientific developments. The aim of our journal is to convey to the public the results of the most interesting and promising research. It is important to note that these articles are united not only by the quality of the performed experimental research and the results obtained, but also by their potential for application in order to improve the quality of people's life.

The article by the authors from Novokuznetsk (**Gorokhova L.G. et al.**) is of the most practical nature: the study proved that indole and 1-benzylindole in case of long-term intake, lead to functional disorders of the cardiovascular system, which are the cause for development of arterial hypertension, coronary heart disease, atherosclerotic vascular lesions. In this regard, at enterprises where there is contact of workers with indole and its derivatives, it is necessary to introduce an expanded list of medical examinations for employees.

As part of a study conducted by authors from Orenburg (**Kazakova T.V. et al.**), it was found that due to environmental pollution, high concentrations

of manganese lead to the accumulation of this microelement in the blood serum, a decrease in the levels of calcium, potassium, magnesium, iron and copper. In the cerebral cortex, the level of manganese, lead, mercury and strontium increases against the background of a decrease in iron and iodine levels, which in turn leads to disorders of the various body systems and the development of various pathological conditions.

The influence of melatonin was studied in two works presented in the current issue: in an article by our regular authors from Novosibirsk (**Michurina S.V. et al.**) and in the article by authors from Volgograd (**Babkov D.A. et al.**). The first group of authors conducted an experiment to identify the effect of melatonin on the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bad, as well as the Bcl-2/Bad ratio in ovarian luteocytes under experimental hyperthermia. Timely administration of melatonin caused a shift in the ratio of Bcl-2/Bad expression areas towards an increase in anti-apoptotic Bcl-2 after only a week of the recovery period and contributed to an earlier normalization of Bcl-2/Bad to physiological levels after two weeks. The second group of authors developed and validated a virtual screening method to identify bioisosteric analogs of melatonin that are promising for study as agents that reduce intraocular pressure. The effectiveness was 40 %, which is a promising indicator for further research and development of the studied compounds as a treatment for glaucoma.

We would like to specifically note the joint article by authors from Yaroslavl and Moscow (**Khokhlov A.L. et al.**) on determining changes in the content of monoamine neurotransmitters and their metabolites in brain structures using high-performance liquid chromatography in combination with tandem mass spectrometry. Until recently, a method for the joint determination of monoamine mediators and their metabolites was not developed. Now it has been fully validated and meets the requirements of Russian and international guidelines. The chosen stabilization method allows samples of brain homogenates to be stored for 30 days after collection.

The study conducted by authors from Moscow (**Adamovskaya O.N. et al.**) can be titled "New times require new research". The authors found that due to the introduction of information and computer technologies into the educational process, schoolchildren, when performing cognitive load on electron ic devices, experience changes in heart rate variability, electrodermal activity and cerebral circulation.

The issue also contains a number of review articles in various areas. For example, a team of authors from Moscow (**Regentova O.S. et al.**) analyzed the prospects for using ultrasound of various intensities to treat patients with malignant brain gliomas. Focused ultrasound is reported to be a promising potential treatment for gliomas. Authors from Ufa (**Ziganshin A.M. et al.**) analyzed the literature on the latest research on post-castration syndrome, its impact on quality of life and correction methods. Despite the high level of prior studies of this problem, there are still significant gaps in reducing the risk of stroke, depression, cognitive disorders and Alzheimer's disease.

Authors from Kostroma (**Tikhonova I.V. et al.**) and Moscow (**Marakshina Yu.A. et al**.) presented articles on psychology. First group of authors examined the perceptions of potential parents about the stressfulness of the parental role (objective and subjective determinants). The second article is devoted to the studying of psychometric properties of the Abbreviated Math Anxiety Scale on a sample of Russian high school students.

The issue also includes articles on topics much more rare for our journal than traditional areas. First, this is an article by authors from Yekaterinburg (**Antonova N.L. et al.**) "Social factors in the formation of eating disorders: Experience of sociological research" presented in the "Demography" section, and an article by the team of authors from Moscow (Siraeva T.V. et al.) in phthisiology "Organizational aspects of medical rehabilitation of patients with respiratory tuberculosis".

More detailed information about the research results of all presented works can be found in the relevant sections of our journal. We hope that presented materials will be of interest to a wide range of readers. We would like to express our gratitude to both the authors and the reviewers who took part in the creation of this issue.

**For citation:** Rychkova L.V. Deputy editor-in-chief's preface to Issue 1, 2024. *Acta biomedica scientifica*. 2024; 9(1): 5-8. doi: 10.29413/ABS.2024-9.1.1

# ПРЕДИСЛОВИЕ ЗАМЕСТИТЕЛЯ ГЛАВНОГО РЕДАКТОРА К № 1 (2024)

**Рычкова**
**Любовь Владимировна**

**Д.м.н., профессор,**
**член-корреспондент РАН**

В первом номере журнала за текущий год экспериментальные исследования занимают лидирующие позиции. Их высокий процент от общего количества публикуемых статей свидетельствует о прогрессивном развитии научных разработок. Задача журнала – донести до общественности результаты самых интересных и перспективных исследований. Важно отметить, что объединяет эти статьи не только качество проведённых экспериментов и полученных результатов, но и их потенциал в применении для улучшения качества жизни людей.

Статья коллектива авторов из Новокузнецка (**Горохова Л.Г. и соавт.**) имеет наиболее практический характер: в рамках исследования было доказано, что индол и 1-бензилиндол в условиях длительного поступления в организм приводят к функциональным нарушениям сердечно-сосудистой системы, которые являются причиной развития артериальной гипертензии, ишемической болезни сердца, атеросклеротических поражений сосудов. В связи с этим на предприятиях, где имеет место контакт с индолом и его производными, необходимо внедрение расширенного списка обследований работников.

В рамках исследования, проведённого авторами из Оренбурга (**Казакова Т.В. и соавт.**), было установлено, что вследствие загрязнения окружающей среды высокие концентрации марганца приводят к кумуляции данного микроэлемента в сыворотке крови, снижению уровня кальция, калия, магния, железа и меди. В коре головного мозга повышается уровень марганца, свинца, ртути и стронция на фоне снижения железа и йода, что в свою очередь ведёт к нарушению функционирования различных систем организма и развитию различных патологических состояний.

Исследование влияния мелатонина представлено сразу в двух исследованиях: в статье наших постоянных авторов из Новосибирска (**Мичурина С.В. и соавт.**) и авторов из Волгограда (**Бабков Д.А. и соавт.**). Первые провели эксперимент по выявлению влияния мелатонина на экспрессию антиапоптотического Bcl-2 и проапоптотического Bad, а также соотношение Bcl-2/Bad в лютеоцитах яичников в условиях экспериментальной гипертермии. Своевременное введение мелатонина показало сдвиг соотношения площадей экспрессии Bcl-2/Bad в сторону увеличения антиапоптотического Bcl-2 уже через неделю восстановительного периода и способствовало более ранней нормализации Bcl-2/Bad до физиологического уровня через две недели. Второй коллектив авторов осуществил разработку и валидацию метода виртуального скрининга для выявления биоизостерических аналогов мелатонина, перспективных для изучения в качестве средств, снижающих внутриглазное давление. Результативность составила 40 %, что является перспективным показателем для дальнейшего изучения и разработки исследуемых соединений в качестве средства для лечения глаукомы.

Хотелось бы отдельно отметить совместную статью авторов из Ярославля и Москвы (**Хохлов А.Л. и соавт.**) об определении изменения содержания моноаминовых нейромедиаторов и их метаболитов в структурах головного мозга с применением метода ВЭЖХ-МС/МС. До недавнего времени методика совместного определения моноаминовых медиаторов и их метаболитов не разрабатывалась. Сейчас же она прошла полную валидацию и соответствует требованиям российских и международных руководств. Выбранный способ стабилизации позволяет хранить образцы гомогенатов мозга в течение 30 дней после отбора.

Исследование, проведённое авторами из Москвы (**Адамовская О.Н. и соавт.**), можно озаглавить как «Новые времена требуют новых исследований». Авторами выявлено, что из-за внедрения в образовательный процесс информационно-компьютерных технологий у школьников при выполнении когнитивной нагрузки на электронных устройствах происходит изменение показателей вариабельности сердечного ритма, электродермальной активности и мозгового кровообращения.

В номере также представлен ряд обзорных статей по различным направлениям. Например, коллективом авторов из Москвы (**Регентова О.**С. и соавт.) проведён анализ перспектив применения ультразвука различной

интенсивности для лечения пациентов со злокачественными глиомами головного мозга. По имеющимся данным, фокусированный ультразвук является многообещающим потенциальным методом лечения глиом. Авторы из Уфы (Зиганшин А.М. и соавт.) выполнили анализ литературы по последним исследованиям посткастрационного синдрома, его влиянию на качество жизни и методам коррекции. Несмотря на высокую степень изученности данной проблемы, всё ещё остаются существенные пробелы в аспектах снижения риска развития инсульта, депрессии, когнитивных расстройств и болезни Альцгеймера.

Авторами из Костромы (**Тихонова И.В. и соавт.**), Москвы (**Маракшина Ю.А. и соавт.**) представлены статьи по психологии. Первый коллектив авторов исследует представления о стрессогенности родительской роли у потенциальных родителей (объективные и субъективные детерминанты). Во второй статье изучаются психометрические свойства сокращённой шкалы математической тревожности на выборке российских старшеклассников.

Также в номер вошли статьи по тематикам куда более редким для нашего журнала, чем традиционные направления. Это статья авторов из Екатеринбурга (**Антонова Н.Л. и соавт.**) «Социальные факторы формирования пищевых расстройств: опыт социологического исследования», вошедшая в раздел «Демографии», и статья коллектива авторов из Москвы (**Сираева Т.В. и соавт.**) по фтизиатрии «Организационные аспекты медицинской реабилитации больных туберкулёзом органов дыхания».

Подробнее с результатами исследования всех представленных работ можно ознакомиться в соответствующих разделах журнала. Надеемся, что представленные материалы будут интересны широкому кругу читателей, за что хотим выразить благодарность как авторам, так и рецензентам, принимавшим участие в создании данного выпуска.

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# INTERNAL DISEASES

## LYSOPHOSPHATIDIC ACID AND ITS RECEPTORS: ROLE IN BRONCHIAL ASTHMA PATHOGENESIS

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### ABSTRACT

*Lysophosphatidic acid (LPA) is a biologically active lipid mediator that regulates a number of signaling pathways involved in the pathogenesis of bronchial asthma. Attention to studying the relationship of LPA with LPA receptors (LPARs) and ion channels with transient receptor potential (TRP) is caused by their role in the initiation and development of bronchial obstruction, which suggests the development of new effective strategies for the treatment of bronchial asthma through blocking LPA synthesis and/or regulation of the activity of the ligand-receptor relationship.*
***The aim of the review.*** *To summarize ideas on the role of lysophosphatidic acid and its receptors in the pathogenesis of bronchial asthma based on the analysis of articles published in English in 2020–2023 from the PubMed database.*
***Conclusion.*** *The review summarizes recent literature data on the chemical structure, biosynthetic pathways and LPA receptors. It presents the information on the role of LPA, LPARs and TRP channels in the pathogenesis of bronchial asthma; summarizes the bronchial asthma therapeutic strategies targeting LPA, LPARs, and TRP channels. The review highlights not only a new perspective on understanding the mechanisms of initiation of asthmatic reactions, but also possible ways to manage them at the stage of correction of their development.*

***Key words:*** *lysophosphatidic acid, lysophosphatidic acid receptors, transient receptor potential channels, bronchial asthma*

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# РОЛЬ ЛИЗОФОСФАТИДНОЙ КИСЛОТЫ И ЕЁ РЕЦЕПТОРОВ В ПАТОГЕНЕЗЕ БРОНХИАЛЬНОЙ АСТМЫ

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## РЕЗЮМЕ

*Лизофосфатидная кислота (LPA, lysophosphatidic acid) является биологически активным липидным медиатором, регулирующим ряд сигнальных путей, вовлечённых в патогенез бронхиальной астмы (БА). Интерес к изучению взаимоотношений LPA с LPA-рецепторами (LPARs, lysophosphatidic acid receptors)и ионными каналами с транзиторным рецепторным потенциалом (TRP, transient receptor potential) обусловлен их ролью в инициации и развитии бронхиальной обструкции, что предполагает разработку новых эффективных стратегий лечения БА через блокирование синтеза LPA и/или регуляции активности лиганд-рецепторного взаимоотношения.*

***Цель обзора.*** *Обобщить представления о роли лизофосфатидной кислоты и её рецепторов в патогенезе бронхиальной астмы на основании анализа статей, опубликованных на английском языке в период с 2020 по 2023 г. в базе данных PubMed.*

***Заключение.*** *В данном обзоре обобщены последние литературные данные о химической структуре, путях биосинтеза и рецепторах LPA. Представлена информация о роли LPA, LPARs и TRP-каналов в патогенезе БА. Обобщены терапевтические стратегии БА, нацеленные на LPA, LPARs и TRP-каналы. Данный обзор подчёркивает не только новый взгляд на понимание механизмов инициации астматических реакций, но и возможные способы управления ими на этапе коррекции их развития.*

***Ключевые слова:*** *лизофосфатидная кислота, рецепторы лизофосфатидной кислоты, ионные каналы с транзиторным рецепторным потенциалом, бронхиальная астма*

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## INTRODUCTION

Lysophospholipids are bioactive lipid mediators localised in cell membranes [1]. They affect cell proliferation, differentiation, survival, migration, adhesion, invasion and morphogenesis, and are associated with neurogenesis, angiogenesis, fibrogenesis and oncogenesis [2]. In recent years, the signalling function of lysophospholipids, in particular lysophosphatidic acid (LPA), has been actively studied in various diseases and pathological conditions. However, not much attention has been paid to the study of the role of LPA in the pathogenesis of bronchopulmonary diseases, particularly bronchial asthma [3-5].

LPA is known to interact with LPA receptors 1-6 (LPARs), peroxisome proliferator-activated nuclear receptors (PPARs), actin-binding proteins (ABPs) and, as recently discovered, ion channel receptors with transient receptor potential (TRP), resulting in the activation of multiple signalling pathways [2, 4, 6, 7].

The role of LPA receptors in the pathogenesis of asthmatic reactions has been actively studied [3], but in recent years the focus of research has shifted significantly to the interaction of LPA with TRP-channel receptors [8, 9]. Malfunction of these channels is known to have a significant impact on the pathogenesis of bronchial obstruction, which makes them potential targets in bronchial asthma treatment [10-18]. LPA has been identified as a ligand for TRP receptors and its ability to modulate the activity of TRPM2 (TRP cation channel, subfamily M, member 2), TRPV1 (TRP cation channel, subfamily V, member 1) and TRPA1 (TRP cation channel, subfamily A, member 1) has been described [3, 19].

After the mechanism of interaction of LPA with LPARs and TRP receptors in the bronchopulmonary system has been established a new perspective on understanding the mechanisms of initiation of asthmatic reactions and possible ways to manage them is being discovered, which suggests fundamentally new possibilities in the development of an effective therapeutic strategy for bronchial asthma at the stage of correction of the development of asthmatic reactions.

Controlling LPA signalling through influencing LPAR1-6 is a relevant pharmacological objective [20]. However, LPA signalling via its receptors is also associated with stimulation of fibrosis development, triggering atherogenesis, oncogenesis and metastasis [21]. Therefore, the use of LPA agonists faces the dilemma of utilising therapeutically effective mechanisms of action of this lipid molecule while avoiding the development of undesirable effects [20], hence the relevance of studying other LPA receptors as therapeutic targets.

This article summarizes recent literature data on the chemical structure, biosynthetic pathways and LPA receptors. The focus is on the role of LPA, LPARs and TRP channels in the pathogenesis of bronchial asthma. Possible therapeutic strategies for bronchial asthma targeting LPAs, LPARs and TRP-channels are summarized and discussed.

The PubMed database was systematically searched for articles published in English in 2020–2023. Five articles published before 2020, which did not include the keywords of this review, were also analyzed to provide more detail on the information provided. The review included information sources that addressed issues relevant to the aim of this review. Information requests included the following set of keywords: 'lysophosphatidic acid', 'asthma', 'transient receptor potential channels', 'lysophosphatidic acid receptors' (Table 1).

The titles of the articles found on request were reviewed and if they matched the literature review topic, the abstracts of the articles were analyzed. If the abstract complied with the inclusion criteria, the full-text version of the article was searched and analyzed.

**TABLE 1**

**RESULTS OF A SYSTEMATIC SEARCH OF ARTICLES IN THE PUBMED DATABASE (2020–2023)**

| Key words | Number of articles for the period 2020–2023 |
|---|---|
| "lysophosphatidic acid" | 698 |
| "lysophosphatidic acid" and "asthma" | 9 |
| "lysophosphatidic acid" and "transient receptor potential channels" | 7 |
| "lysophosphatidic acid" and "asthma" and "transient receptor potential channels" | 1 |
| "asthma" and "transient receptor potential channels" | 67 |
| "lysophosphatidic acid" and "lysophosphatidic acid receptors" | 304 |
| "lysophosphatidic acid" and "asthma" and "lysophosphatidic acid receptors" | 5 |
| "asthma" and "lysophosphatidic acid receptors" | 4 |

## 1. LYSOPHOSPHATIDIC ACID

Lysophosphatidic acid is classified as a lysoglycerophospholipid, a phospholipid with only one fatty acid residue, unlike glycerophospholipids that have two fatty acids at the sn-1 and sn-2 positions [6].

Lysophosphatidic acids are represented by different molecular species depending on the presence of saturated or unsaturated fatty acid residues in their structure (LPA16:0, LPA18:0, LPA18:1, LPA18:2, LPA20:4 and LPA22:6). Also, the structure and activity of LPA are determined by the position of fatty acids in the glycerol molecule [1, 6] (Fig. 1).

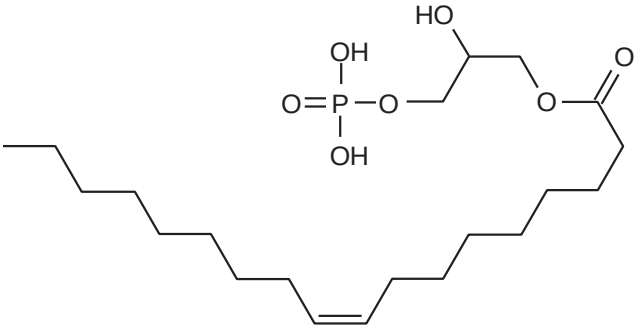


***FIG. 1.***
*Chemical structure of lysophosphatidic acid*

LPA biosynthesis is carried out through several pathways [2, 6, 7] (Table 2).

In the first pathway, LPA biosynthesis depends on the activity of phospholipases [22]. This pathway includes cleavage of membrane phospholipids (phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine) or cleavage of diacylglycerol (DAG) and phosphatidic acid formation. Phosphatidic acid is a substrate for phospholipases A1 and A2, which release fatty acids from the sn-1 or sn-2 positions, respectively, forming LPA.

Phospholipase A1 acts both extracellularly and intracellularly. Extracellular phospholipase A1 is involved in triacylglycerol hydrolysis and fatty acid cleavage. Phospholipase A2 is a large superfamily comprising 15 groups and 30 isoforms belonging to four types: secreted (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB), cytosolic, calcium-independent and lipoprotein-associated phospholipases A2. Phospholipase A2 hydrolyses unsaturated fatty acids and is involved in the generation of eicosanoids and platelet-activating factor. On the one hand, secreted phospholipase A2 releases omega-3-polyunsaturated fatty acids (PUFAs), which are a substrate for anti-inflammatory and pro-resolving oxylipins. On the other hand, secreted phospholipase A2 is involved in the generation of fatty acids, lysophosphatidic acid, lysophospholipids, prostaglandins, leukotrienes and thromboxanes, which have pro-inflammatory effects [1, 22].

In the second pathway, LPA biosynthesis is mediated by autotaxin (ATX) [6]. Autotaxin (isoforms ATX-α, ATX-β, ATX-γ, ATX-δ and ATX-ε) or lysophospholipase D belongs to the ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) family. The transcription of the *ENPP2* gene located in the human chromosomal region 8q24 is regulated by several pro-inflammatory and transcription factors [23]. ATX inhibition leads to decreased levels of pro-inflammatory mediators (tumour necrosis factor, interleukin (IL) 1, IL-6) in the experiment [24].

ATX is active in most biological fluids, including serum/plasma, bronchoalveolar lavage (BAL) fluid, cerebrospinal fluid and urine. In platelets, autotaxin can bind to platelet integrins αVβ3 and αIIbβ3. Adipose tissue is considered to be the main source of autotaxin in blood [6].

LPA is also formed from glycerol-3-phosphate with the participation of glycerol-3-phosphate acyltransferase (GPAT). Lastly, LPA can be cleaved to monoacylglycerol and diacylglycerol with the participation of lysophosphatases, to PA – via LPA-acyltransferase, and to G3P – with the participation of lysophospholipases [6].

### Lysophosphatidic acid receptors

The physiological role of circulating LPAs is to preferentially transmit signals by interacting with transmembrane G-protein coupled receptors (GPCRs), which include LPA receptors (LPAR1–6) [2, 6]. Furthermore, recent studies revealed that LPA is a ligand for some TRP channels [2, 4, 6, 7]. The LPA receptors are summarised in Table 3.

LPAR1–3 belong to the endothelial differentiation gene (EDG family: LPAR1/EDG2, LPAR2/EDG4 and LPAR3/EDG7. LPAR4 (P2RY9, GPR23), LPAR5 (GPR92) and LPAR6 (P2RY5, GPR87) belong to purinergic receptors (P2Y).

**TABLE 2**

**PATHWAYS AND ENZYMES OF LYSOPHOSPHATIDIC ACID BIOSYNTHESIS**

| Pathways of LPA biosynthesis | Enzymes involved in LPA biosynthesis |
|---|---|
| Phosphatidic acid | PLA1, PLA2 |
| Lysoglycerophospholipids (LPC, Lyso-PS and LPE) | ATX/Lyso PLD |
| Glycerol 3-phosphate (G3P) | GPAT |

Recent studies have shown that LPA can also activate P2Y10 [2].

LPAR1 is widely expressed in various organs and tissues, but mainly in the brain, heart, placenta, large and small intestines. Higher expression of LPAR2 mRNA is found in kidney, uterus, and testis; lower expression – in thymus, pancreas, and spleen. The level of LPAR3 mRNA is higher in the heart, lungs, pancreas, brain, prostate and ovaries. LPAR4 mRNA levels are elevated in mouse skin, heart, ovaries, and thymus. Large amount of LPAR5 is expressed in the spleen, large and small intestines. LPAR6 is associated with hair growth, but there are relatively few studies on its role and mechanisms in various systems [2].

LPARs activate Gα protein subtypes (Gα12/13, Gαq/11, Gαi/o and Gαs) that modulate downstream signalling pathways. Thus, the ROCK (Rho/Rho-associated protein kinase) signalling pathway is activated via Gα12/13; Gαq/11 activates phospholipase C (PLC) and further downstream cascades, GSK3 (glycogen synthase kinase 3) and CREB; Gαi/0 activates the PLC pathways, CREB and GSK3, and stimulates extracellular signal-regulated kinases 1/2 (ERK1/2), Akt/phosphoinositide-3-kinases (PI3K, PI3-kinases) and inhibits cyclic adenosine monophosphate (cAMP) production; GαS modulates adenylate cyclase and protein kinase A (PKA) activity by activating the cAMP signalling pathway [25].

**TABLE 3**

**LYSOPHOSPHATIDIC ACID RECEPTORS**

| LPA receptors | Signalling pathway/effector |
|---|---|
| LPAR1/EDG2 | Gα12/13–Rho–ROCK<br>Gαi/o–Ras–Erk 1/2<br>Gαi/o–PI3K–Akt-mTOR<br>Gαi/o–PLC–CREB и GSK3<br>Gαq/11-PLC–CREB и GSK3 |
| LPAR2/EDG4 | Gα12/13–Rho–ROCK<br>Gαi/o–Ras–Erk 1/2<br>Gαi/o–PI3K–Akt-mTOR<br>Gαi/o–PLC–CREB и GSK3<br>Gαq/11–PLC–CREB и GSK3 |
| LPAR3/EDG7 | Gαq/11–PLC–CREB и GSK3<br>Gαi/o–Ras–Erk 1/2<br>Gαi/o–PI3K–Akt-mTOR<br>Gαi/o–PLC–CREB и GSK3 |
| LPAR4 (P2RY9, GPR23) | Gα12/13–Rho–ROCK<br>Gαq/11–PLC–CREB и GSK3<br>Gαi/o – Ras – Erk 1/2<br>Gαi/o–PI3K–Akt-mTOR<br>Gαi/o–PLC–CREB и GSK3<br>Gαs–AC–RKA |
| LPAR5 (GPR92) | Gα12/13–Rho–ROCK<br>Gαq/11–PLC–CREB и GSK3<br>Gαs–AC–RKA |
| LPAR6 (P2RY5, GPR87) | Gα12/13–Rho–ROCK<br>Gαi/o–Ras–Erk 1/2<br>Gαi/o–PI3K–Akt-mTOR<br>Gαi/o–PLC–CREB и GSK3<br>Gαs–AC–RKA |
| P2RY10 | – |
| TRPV1 | Direct interaction with K710 at the COOH end |
| TRPA1 | LPAR5-PLD<br>Direct interaction with intracellular KK672-673 and KR977-978 |
| TRPM2 | LPAR1-Gαi/o-MAP kinases-PARP-1-ADP-ribose |

The main effect of LPA is its ability to modulate the actin cytoskeleton through the activation of small GTPases of the Ras superfamily via Gαi/o (stimulation of extracellular Erk1/2 signals) and Rho via Gα12/13 (stimulation of ROCK) [26]. These receptors signal through MARK, PLC and tyrosine kinases and initiate the synthesis of a number of transcription factors that interact with DNA sites and initiate proliferation, cell migration or intercellular interactions.

LPA mediates many functions through LPARs, in particular $Ca2^{+}$ mobilisation, survival, proliferation, adhesion, cell migration, immune function, by reducing chemokine production and inhibiting cell migration, and myelination [2].

**TRP channels**

As mentioned above, lysophosphotidic acid actively interacts with TRP-channels, which play an essential role in sensory physiology (smell, taste, vision, touch, thermo-sensing, osmosensing), pathophysiology of pain and inflammation, and act as signalling conductors in cells [27-29]. The function of these channels is to alter the cell membrane potential and fluctuate the intracellular concentration of free calcium $[Ca2^{+}]i$ in response to stimulating environmental influences [30]. TRP channels integrate TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), TRPP (polycystin) receptors [28-33]. TRPA1, TRPV1, TRPV2, TRPV4, TRPM3 and TRPM8 are thermosensory TRP-channels that are activated by changes in temperature, particularly ambient temperature [27, 32]. TRPVs are activated by heat, while TRPA1 and TRPM8 are activated by cold [33, 34].

TRP-channels are expressed in neuronal cells as well as in cells of the respiratory tract [14, 27]. To date, it has been established that LPA is able to directly or indirectly activate TRPM2, TRPV1 and TRPA1 [3, 35]. For instance, TRPM2 and TRPA1 are activated indirectly. Extracellular LPA elevation activates LPAR5, phospholipase D (PLD, phospholipase D) leading to increased intracellular LPA levels and TRPA1 activation. Activation of LPAR5 via Gαi/o stimulates PARP-1 (poly(ADP-ribose) polymerase 1), ADP-ribose, and TRPM2. TRPA1 and TRPV1 are directly activated [3, 35].

Thus, literature data demonstrate that LPA realizes its mechanism of action through interaction with LPARs and TRP channels, activating a number of signaling pathways. LPARs and TRP-channels are widely represented in the bronchopulmonary system, which makes it important to further study the effects of LPA in BA, despite the fact that clinical trials of TRP modulators have not been successful so far [14].

## 2. WHEEZING AND TRP-CHANNELS

Epidemiological studies of recent years convincingly demonstrate that the combination of high, low temperatures and humidity is accompanied by the development of wheezing in BA patients [12], which is mediated by the participation of TRP-channels in the reception of physicochemical stimuli of the environment [10], in particular, thermosensory TRPA1, TRPV1, TRPV2, TRPV4, TRPM3 and TRPM8 [27, 33].

Strong evidence has been presented for the important role of TRPA1, TRPC6, TRPM2, TRPM5, TRPM7, TRPM8, TRPV2, TRPV4 in airway function and pathogenesis of related diseases [13, 15-17].

For example, C-fibres, non-neuronal cells, airway cells, smooth muscle cells, epithelial cells and fibroblasts express TRPA1 [36]. Cigarette smoke, car exhaust, air pollution, reactive oxygen intermediates (ROIs), and various temperature conditions are among the known TRPA1 agonists [37, 38]. Activation of sensory nerves via TRPA1 initiates coughing, mucus secretion, airway hyperresponsiveness, inflammation and development of wheezing [15, 17, 37, 39].

Vanilloid receptors (TRPV1-6) are localised in nociceptive neurons, airway sensory fibres, on bronchial epithelial cells, mast cells, macrophages and human airway smooth muscle cells [14, 15, 28].

TRPV1 is present in airway sensory fibres lining the trachea, bronchi and alveoli, and is also expressed in bronchial epithelial cells and in intrapulmonary arterioles. The relationship between TRPV1 and bronchopulmonary diseases has been demonstrated *in vitro* and *in vivo*. Activation of TRPV1 by agonists leads to the release of neurokinin A, substance P and CGRP, which contribute to smooth muscle contraction, mucus hypersecretion, coughing and the development of asthma-like symptoms [13]. Of significance, a role for TRPV1, which is mainly expressed in neurons, in IL-33 secretion by airway epithelium in response to exposure to the house dust mite allergen HDM and fungal allergens has recently been described [14]. Bronchospasm and asthma-like symptoms that develop in response by exposure to cold air and high humidity are associated with the involvement of not only TRPV1 but also TRPV2 and TRPV4 in osmoreception [16, 40]. TRPV1 and TRPV4 seem to contribute most significantly to the development and exacerbation of BA [18].

TRPA1 channels often act in concert with TRPV1 [41]. These data suggest that the interaction between TRPA1 and TRPV1 may be essential in regulating the function and excitability of pulmonary sensory neurons during airway inflammation. TRPV1 can also oligomerize with other TRP family subunits, including TRPV3 [41].

TRPV1-4 are channels showing a predominance of $Ca2^{+}$ influx over $Na^{+}$ influx, with TRPV5 and TRPV6 being $Ca2^{+}$ – only permeable channels [18]. In a review by J.H. Nam and W.K. Kim the relationship between TRP channels and immune cells involved in the pathogenesis of allergic diseases, as well as therapeutic agents targeting these channels are discussed [30]. An increase in intracellular $Ca2^{+}$ concentration causes the release of histamine, anaphylactic chemotaxis factor of eosinophils and neutrophils from mast cells and leads to contraction of bronchial musculature. This intracellular $Ca2^{+}$ signalling is provided by TRP-channels involved in almost all types of immune cells, in particular mast cells, T-cells and B-cells involved in the pathogenesis of allergic inflammation characteristic of allergic BA [30].

## 3. LYSOPHOSPHATIDIC ACID, LPARS AND TRP-CHANNELS IN THE PATHOGENESIS OF WHEEZING

LPA synthesis is enhanced by inflammation, particularly localised in the bronchopulmonary system [5, 42-46]. *In vitro* studies have revealed that LPA activates eosinophils, lymphocytes, mast, dendritic, epithelial and smooth muscle cells in the airways.

LPA levels have been demonstrated to be significantly elevated in the BAL of patients with BA [5]. Of interest, LPA has been identified as a regulator of the epithelial-mesenchymal transition involved in the conversion of fibroblasts to myofibroblasts and the development of airway remodelling [47].

The functions of LPA in the bronchopulmonary system are conditioned by its reactions with LPARs [2, 5, 46] and TRP-channels [4, 8]. As previously mentioned, central to the effects of LPA is its ability to activate the small GTPases Ras (Erk1/2 stimulation) and Rho-kinases via Gα12/13 (ROCK stimulation) [26].

The Rho-kinase of the ROCK signalling pathway is known to play a key role in maintaining the expression of muscle contractions during smooth muscle activation. Rho-kinase inhibition is currently being studied as a component of combined treatment of wheezing in BA [48]. Accordingly, the LPA – LPARs – ROCK signalling pathway requires to be studied closely.

LPA via LPAR1-3 activates p38 MAPK and JNK kinases and induces IL-8 production, which increases inflammation and promotes airway remodelling in BA [5]. These data are evidence that LPA plays an important role in allergic airway inflammation and that blockade of LPARs may have therapeutic potential in BA [5].

LPA is able to activate TRPA1, TRPM2 and TRPV1 [3, 17]. Currently, only sporadic works have focused on the interaction between LPA and TRPA1 in various pathologies [9]. TRPM2 are expressed in pulmonary endothelium and are involved in the regulation of barrier function, cell death, cell migration and angiogenesis [16]. Although LPA is able to activate TRPM2 [3], their interaction in BA has not been studied [17].

Recently, it has been described that LPA can activate TRPV1 [3, 19]. M. Benítez-Angeles et al. reported that LPA directly interacts with TRPV1 through the K710 residue in the C-terminal of TRPV1 [19].

Interestingly, LPA has been implicated in the pathogenesis of wheezing through interaction with LPAR and TRPV1 [3, 4]. A series of works by N.G. Jendzjowsky et al. was devoted to the study of the role of carotid bodies in the occurrence of wheezing [3]. Carotid corpuscles respond to changes in partial pressure of oxygen, carbon dioxide, pH, temperature, and have also demonstrated the ability to react in response to bacterial infection [49] and exposure to allergens [3]. N.G. Jendzjowsky et al. have revealed that the increase in blood LPA caused by allergen exposure activates carotid cells and causes wheezing via LPAR and TRPV1 [3]. This signalling pathway involves PKCε (protein kinase C epsilon) binding LPAR1 and TRPV1 to each other. In their recent work, N.G. Jendzjowsky et al. also have revealed that repeated exposure to allergen increases carotid body sensitivity to LPA as a consequence of LPARs hyperexpression in carotid bodies. These experimental data demonstrate the ability of allergens to sensitise carotid cells, highlighting their role in the development of BA and the involvement of the LPAR1 – PKCε – TRPV1 pathway in the pathogenesis of asthmatic reactions [3]. However, it is worth noting that this mechanism has not yet been confirmed in humans.

In summary, the presented data evidence the therapeutic potential of LPA, TRP-channels and LPARs, which play a definite role in the development of airway inflammation and bronchospasm in BA.

## 4 MODERN THERAPEUTIC APPROACHES TO THE REGULATION OF LPA ACTIVITY

### LPAR antagonists

LPA as a potent signalling molecule affects numerous physiological and pathological processes; therefore, the control of LPA signalling is of growing pharmacotherapeutic interest worldwide [20]. The action of LPA is mediated through the activation of several types of molecular targets, including LPAR1-6, which are now targeted by most drug development methods in a wide range of pathologies [20]. LPA signalling through its receptors, however, is also associated with the development of pathological responses that include, for example, stimulation of fibrosis or the development of atherogenesis, which should be taken under consideration in drug development [20, 21].

In a brilliant review by S. Llona-Minguez et al. the results of 30 years of studies conducted in the pharmaceutical industry in relation to LPA and its receptors have been summarised [50]. The co-authors of the review note that LPAR1 and LPAR1/LPAR3 antagonists have attracted the most attention for pharmaceutical development (Kirin Ki16425). Of the two potential LPAR antagonist molecules (BMS-986020 for the treatment of idiopathic pulmonary fibrosis and SAR-100842 for the treatment of systemic sclerosis), the study of SAR-100842 was discontinued [6]. S. Llona-Minguez et al. also analyse a number of developmental issues: for example, the lack of potent and selective low molecular weight LPAR3 and LPAR5 agonists, LPAR4 antagonists and the lack of LPAR6 modulators [50]. The authors also identified a wide range of conditions in which selective LPA modulators may be effective (fibrosis, thrombosis, cancer metastasis, urinary tract diseases, and several others), while emphasising the inherent risk of side effects and the need to develop new LPA modulators with selectivity in mind. Also S. Llona-Minguez et al. emphasize the need to detail the structure of LPA receptors and to develop the design of new drugs on this basis [50].

Y.J. Lee et al. indicate the prospect of developing LPAR2 antagonists for the BA treatment [51]. The authors of this study compared the effects of an antagonist (H2L5186303) and an agonist (GRI977143) of LPAR2 in an experimental protocol for ovalbumin-induced allergic BA (OVA).

H2L5186303 demonstrated reduced airway hyperresponsiveness, decreased levels of inflammatory cytokines, mucin production and eosinophil counts. The authors of this study suggest that the development of LPAR2 antagonists will achieve greater therapeutic efficacy in BA compared to the action of agonists in this pathology [51].

M. Kondo et al. also demonstrated on the model of allergic BA that administration of LPAR2 antagonist (H2L5186303) effectively suppressed allergic inflammation [5]. The authors revealed that the increase in IL-13 production as a result of LPA stimulation was inhibited by treatment with LPAR2 antagonists. The authors of this study also demonstrated that LPA exacerbates allergic bronchial inflammation by promoting Th2 differentiation and IL-33 production, whereas the LPAR2 antagonist controls IL-33 production. According to the conclusion of M. Kondo et al. blockade of LPAR2 may be an effective therapeutic strategy in BA [5].

N.G. Jendzjowsky et al. demonstrated that administration of LPA receptor antagonist (BrP-LPA) effectively blocks bronchoconstriction in experiment [3].

### Drugs inhibiting synthesis or enhancing degradation of LPA

Many therapeutic agents are currently available that inhibit LPA synthesis by affecting the reduction of autotoxin activity or enhancing LPA degradation [43, 51-53].

There is currently sufficient evidence that the ATX-LPA axis is involved in the processes of cancer initiation and metastasis, the development of atherosclerosis, obesity, arthritis, glaucoma, acute and chronic liver failure, fibrosis of the liver, kidneys and lungs and many other diseases and pathological conditions [21, 23, 44, 54, 55]. Some researchers continue to support and develop the idea that this axis plays an important role in the development of airway inflammation [21], particularly in BA [5, 45]. For example, the role of the ATX-LPA axis in lung development, lung function in norm and pathology is brilliantly summarised in a recent study by S. Zulfikar et al. [21].

One possible method to affect the LPA signalling pathway is through ATX inhibition [45, 52, 56, 57]. ATX inhibitors may be effective for the treatment of chronic inflammation [44, 52, 58]. New imidazo[1,2-a]pyridine derivatives are considered as potent allosteric inhibitors of ATX. Their promising antifibrotic efficacy was demonstrated in a mouse lung model [59]. J.W. Cuozzo et al. found inhibition of LPA production through the interaction of compound 1 (X-165) with autotaxin under experimental conditions. This compound has also demonstrated efficacy in a mouse model of fibrosis [53].

It is conceivable that ATX is a relatively safe therapeutic target, but to date there is insufficient information about its safety in humans [45]. No ATX inhibitors are currently approved by the US Food and Drug Administration (FDA), with only two drugs in clinical trials, BBT-877 and BLD-0409 [52]. ATX inhibitor researchers concur that there is a need to optimise their kinetic properties and to develop inhibitors with multiple targets. For example, in LPA-mediated diseases, ATX, PLA, and PPAR may serve as targets [52].

### TRPV receptor antagonists

As mentioned above, LPA is able to activate TRP-channels (TRPA1, TRPM2 and TRPV1); some of them are involved in the pathogenesis of wheezing [3]. These experimental data demonstrate the ability of allergens to sensitise carotid cells and activate the LPAR1 – PKCε – TRPV1 pathway, which plays an important role in the pathogenesis of asthmatic reactions. Since administration of a TRPV1 receptor antagonist (AMG9810) blocks the development of wheezing, vanniloid receptors may be an important target for therapy of BA [3].

Thus, there are now a number of LPAR antagonists, inhibitors of LPA synthesis, and drugs that enhance LPA degradation that are effective in BA. In addition, there is evidence that TRPV1 receptor antagonists are promising for the treatment of wheezing.

## CONCLUSION

LPA controls many physiological processes in the cell and is one of the mediators whose expression is enhanced in inflammation localised in the bronchopulmonary system. LPA receptors have been revealed to be activated by a number of downstream signalling pathways through interactions with LPARs, nuclear receptors and TRP-channels. Although LPARs are potent activators of signalling pathways, the study of TRP-channels also deserves close attention because of their involvement in the pathogenesis of bronchial obstruction.

As evident from the literature provided, some ATX and LPA antagonists reduce airway inflammation and hyperresponsiveness underlying the pathogenesis of BA. A number of studies also point to the promise of developing LPA receptor antagonists (particularly LPAR2) for the treatment of BA. In addition, there is emerging evidence that TRPV1 receptor antagonists are promising for the management of wheezing. Recent studies also reveal that LPA is involved in the pathogenesis of wheezing through interactions with LPAR and TRPV1, which offers interesting prospects for the development of inhibitors with multiple targets. A number of researchers have indeed emphasised the need not only to optimise the kinetic properties of ATX inhibitors, but also to develop inhibitors with multiple targets for their action. For example, in LPA-mediated diseases, ATX, PLA and PPAR may serve as multiple targets. Based on the analysed literature, we can also assume that such multiple targets for the development of LPA inhibitors may be LPAR and TRP-channels, which will allow to effectively influence the main links in the wheezing pathogenesis. The purpose of this review was to draw researcher attention to this area, which undoubtedly requires further study.

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**Conflict of interest**

The authors of this article confirmed that there is no conflict of interest to be reported.

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## DEMOGRAPHY

# SOCIAL FACTORS IN THE FORMATION OF EATING DISORDERS: EXPERIENCE OF SOCIOLOGICAL RESEARCH

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## ABSTRACT

***Background.*** *The increasingly complex structure of modern society and systemic changes associated with the processes of digitalization and mediatization are raising the issue of preserving the health of the younger generation, which is the most receptive and vulnerable group to external impact.*
***The aim of the study.*** *Based on survey data, to carry out an analysis of social factors influencing the development of eating disorders in girls.*
***Materials and methods.*** *The method of collecting information was an online survey of girls aged 18 to 25 years old, conducted in Yekaterinburg in the spring of 2023 (n = 205). Using in-depth interviews, 8 girls were interviewed to clarify the social factors in the formation of eating disorders.*
***Results.*** *In girls' assessments, distorted body image, fear of weight gain, and orthorexia are the signs of eating disorders. Social factors influencing the formation of an ideal body image and eating disorders are social media, circle of contacts, as well as the external environment, which causes psychological and physical stress. Despite the negative perception of the content of communities promoting anorexic bodies, girls are well aware of the published materials. The main tool for achieving the standard model of a girl's physicality is dietary practices, food restrictions and calorie counting, despite the recognition of the negative consequences of their use.*
***Conclusion.*** *Eating behavior for girls is one of the leading tools for achieving an ideal body, ideas about which are formed today by social media, as well as the immediate social environment, including the opposite sex. Communities on social networks can serve as a source for development of deviant eating attitudes, on the one hand, and a real driving force in providing assistance and support to young people with signs of eating disorders, on the other hand. We believe that online consultations with specialists and the development of a parental culture of health protection can have a significant impact on the development of harmonious, health-protecting eating habits.*

***Key words:*** *eating behavior, eating disorders, social factors, social media, circle of contacts, ideal female body, youth*

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# СОЦИАЛЬНЫЕ ФАКТОРЫ ФОРМИРОВАНИЯ ПИЩЕВЫХ РАССТРОЙСТВ: ОПЫТ СОЦИОЛОГИЧЕСКОГО ИССЛЕДОВАНИЯ

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## РЕЗЮМЕ

***Обоснование.*** *Усложняющаяся структура современного общества и системные изменения, связанные с процессами цифровизации и медиатизации, актуализируют вопрос о здоровьесбережении молодого поколения, которое является наиболее восприимчивой и уязвимой к воздействиям внешней среды группой.*
***Цель исследования.*** *На основе опросных данных осуществить анализ социальных факторов, влияющих на формирование у девушек расстройств пищевого поведения.*
***Материалы и методы.*** *Методом сбора информации стал онлайн-опрос девушек от 18 до 25 лет, проведённый в Екатеринбурге весной 2023 г. (n = 205). С использованием глубинных интервью было опрошено 8 девушек для уточнения социальных факторов формирования отклонений в пищевом поведении.*
***Результаты.*** *В оценках девушек искажённое восприятие тела, страх набора веса, а также орторексия являются признаками пищевых расстройств. Социальными факторами, влияющими на формирование идеального образа тела и расстройств пищевого поведения, выступают социальные медиа, контактный круг, а также внешняя среда, которая вызывает психологическое и физическое напряжение. Несмотря на негативное восприятие контента сообществ, пропагандирующих анорексичное тело, девушки прекрасно осведомлены о публикуемых материалах. Основным инструментом достижения эталонного образца телесности девушки выступают диетарные практики, ограничения в еде и подсчёт калорий, несмотря на признание негативных последствий их использования.*
***Заключение.*** *Пищевое поведение для девушек выступает одним из ведущих инструментов достижения идеального тела, представления о котором формируют сегодня социальные медиа, а также ближайшее социальное окружение, в том числе противоположного пола. Сообщества в социальных сетях могут служить источником становления девиантных пищевых установок, с одной стороны, и реальной движущей силой в оказании помощи и поддержки молодёжи с признаками расстройства пищевого поведения – с другой. Полагаем, что онлайн-консультации специалистов и развитие родительской культуры здоровьесбережения могут оказать значительное влияние на становление гармоничных здоровьеразвивающих пищевых привычек.*

*Ключевые слова: пищевое поведение, расстройства пищевого поведения, социальные факторы, социальные медиа, контактный круг, идеальное женское тело, молодёжь*

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## INTRODUCTION

Eating disorders are a serious and intractable problem in modern health care systems. According to J.F. López-Gil et al. the overall proportion of children and adolescents with eating disorders is 22.36 %, with girls significantly more likely to report eating disorders than boys [1].

Eating disorders (EDs) refers to a disorder of eating, expressed as a lack of ability to control timely and correct intake [2]. The main types of eating disorders include anorexia nervosa, bulimia nervosa, and compulsive overeating [3], which often lead to secondary comorbidities. The main factors in the formation of anorexia nervosa are distorted perception of one's body and false interpretation of others' attitudes – dysmorphophobia [4], which develops in adolescence, and most often in girls [5]. Patients with anorexia and bulimia have depressive episodes [6], and the course of the diseases is long-standing/chronic with possible remissions [7]. Regarding compulsive overeating, excessive food intake is associated with the desire to reduce emotional stress [8].

Acting as one of the complex biopsychosocial phenomena of human life, the formation of routinised everyday practices of eating behaviour originates from an early age and is influenced by a wide range of institutions and agents. In particular, parents – agents of primary socialisation – have a great influence in forming children's food habits both through their own food attitudes and through control over the child's diet, which is implemented from the position of restricting "unhealthy food" in general and forcing to use exclusively healthy foods [9]. This can form the attitude of eating "in reserve" in the absence of feeling hungry. Peers included in the contact circle also exert pressure: ridicule from peers causes dissatisfaction with the body and the desire to change it by restricting food intake [10].

In the process of socialisation, eating behaviour becomes a tool for implementing a variety of functions, acquiring sign-symbolic properties. In essence, eating behaviour marks the social position of an individual and becomes a reflection of his or her style and lifestyle, in which orientation to standardised beauty standards is not the least important. German researcher T. Fuchs states that a person who tries to get in shape to meet modern standards has a disturbed body image, experiences increased anxiety and shame [11].

Modern media actively broadcast ideal body images. Researchers have revealed that an increase in eating disorders is associated with women's weight loss in movies and magazines [12]: women in TV shows and movies between the ages of 21 and 39 are more likely to be portrayed as thin and wearing revealing clothing than women of other ages, and female characters with larger and heavier bodies are virtually absent from the screen in family films on prime-time television [13]. Digital Influencers [14], which establish models of the ideal body (using special tools (retouching, filters, etc.)), creating standards and proposing mechanisms for its construction, including through dietary practices, are becoming increasingly important for the young generation.

## THE AIM OF THE STUDY

To reveal social factors influencing the formation of eating disorders in girls on the basis of survey materials.

## MATERIALS AND METHODS

The sociological study was conducted in the spring of 2023 in the city of Yekaterinburg. Using an online survey, a questionnaire was conducted with 205 girls aged 18 to 25: 49.8 % of the respondents had higher education; 2/3 of the girls said that at the time of the survey they were in a romantic relationship with the opposite sex, and 8 % had already married. We emphasize that 39 respondents with a confirmed diagnosis from the eating disorder spectrum participated in the survey. The remaining girls confirmed some difficulties in the practices of implementing healthy/harmonious/adequate eating behaviors to the screening question of the questionnaire. The author's questionnaire contained 45 questions (closed, open, semi-closed). The obtained materials were processed using the Vortex program (Shkurin D.V.). To deepen the data obtained, we also conducted 8 in-depth interviews with girls (Table 1), the materials of which were transcribed and summarized in accordance with the research objectives.

The study was of a pilot nature and its results cannot be extended to the entire population of Russian girls, but they demonstrate problem areas in the development of young women's eating behaviour and reveal the social factors that influence the formation of deviations in eating behaviour. The authors aim to draw the attention of practitioners and managers to current issues in the field of health care for the younger generation in general and reproductive health of the female community in particular.

## RESULTS AND DISCUSSION

The results of the study reveal that the signs of eating disorders, as assessed by the girls, are, first of all, distorted perception of their bodies, fear of gaining weight and orthorexia, i.e. division of foods into "healthy" and "harmful" (table 2).

The ideal body model is interpreted by the respondents in a very wide range: from "hourglass" (25.7 per cent) to anorexic physique (24.9 per cent), with 68.3 per cent of the surveyed girls striving to meet the latter standard. The results of in-depth interviews also testify to the expansion of the boundaries of body standards: «*In fact, now, as I think, the standards are becoming more*

**TABLE 1**

**TRANSCRIBED AND SUMMARIZED DATA FROM IN-DEPTH INTERVIEWS WITH GIRLS**

| The informant's code | Age | Category |
|---|---|---|
| I1 | 23 | Employed |
| I2 | 22 | Student |
| I3 | 22 | Student |
| I4 | 23 | Employed |
| I5 | 23 | Employed |
| I6 | 24 | Employed, on maternity leave |
| I7 | 24 | Employed |
| I8 | 23 | Employed |

*and more "blurred"* [I2]; «*Now skinnies, heroin chic, thinness is coming back into fashion again*» [I5]; «*There are always a lot of complaints about girls. Something that will probably never go out of style is thinness. A skinny body always looks more aesthetic*» [I7];»*Well, skinny girls are always in fashion. An athletic body, too. What will always be in fashion is a slim waist, long legs, those golden 90-60-90*» [I8].

According to studies, impaired body perceptions increase the risk of developing eating disorders [15]. Among the main daily practices aimed at matching one's own body to ideal standards, eating behaviour takes the lead. Specifically, 29.2 per cent of those surveyed plan to limit their food intake, and 40.7 per cent of them have used diets and calorie counting. M. Foucault believed that in any society the human body is subjected to the discipline of obedience and certain manipulations [16]. Concurrently, according to the girls questioned, food restrictive disciplinary practices aimed at developing an ideal body image lead to negative consequences for both physical and mental health (84.1 per cent). The most common are breakdowns after diets, fear of weight gain, obsessive thoughts about food, and deterioration in mental health (Figure 1).

In our questionnaire, we asked female respondents to assess the factors that influence perceptions of the ideal body and patterns of eating behaviour as a tool for achieving conformity to reference body image patterns. According to the respondents, the sources of stereotypes about the ideal female body are primarily social media: accounts of media personalities in social networks (24.3 %), as well as visual materials (photos/videos) posted in communities (19.8 %). According to the data obtained during the interviews, various body images circulate in the Internet space, which for young people become standards: «*It's definitely social networks in the first place*» [I1]; «*...groups in VK (VKontakte)...*» [I5]; «*...the Internet, ... fitness bunnies, models, bloggers*» [I7]. Social media today is the most powerful conveyor belt of production and reproduction of appearance culture, including the tyranny of slenderness (in the rhetoric of I.V. Sokhan [17]), the perception of the body as a project and gender stereotypical body ideals. According to informants' estimates, «*overweight girls are not considered acceptable, let alone girls and women, for example, with a size 50+*» [I4], since «*there is an ideal available in the Internet, and if you do not correspond to it, you are something «not like that», i.e. immediately there is judgement, and you have to "adjust" yourself to the standards*» [I3], as «*there is a feeling of unworthiness, we become dissatisfied with our body and face, which causes ED*» [I5].

The specificity of modern media is that individuals not only act as consumers of content, but also produce social reality by participating in discussions, reposts, and copywriting. Girls (89.8 %) surveyed confirmed that they are/were subscribed to communities in the social network VK (VKontakte), the main topics of which are unhealthy weight loss («40 kg», «Typical anorexic», «Fat logic», «Psychology of anorexia», «Fat anorexic»). At the same time, 78.8 % of respondents answered that they have a negative attitude towards them.

The interview texts also include recognition of the content offered by community members and negative attitudes towards them: «*I am familiar with the 40 kg community, all my friends used to subscribe to it. I sometimes went there, just to look, but I was not subscribed*' [I2]; «*I have never been a member myself, but I have a lot of girls I know who have «stayed» there and from whom I have heard stories about these groups. Well, maybe a couple of times I went there just for the sake of interest*» [I6]; «*I have a negative attitude towards such communities, because they promote unhealthy thinness, which can be very harmful to the body. ...Especially now ED is very popular among teenagers*» [I4]; «*they form the wrong type of figure..., ...ambassadors of thinness..., ...of course, this is in the mind of a child or teenager. And if you don't look like that, then you spend the rest of your life thinking that something is wrong with you*» [I5]; «*it seems to me*

**TABLE 2**

**SIGNS OF EATING DISORDERS EXPERIENCED BY FEMALE RESPONDENTS (% OF RESPONDENTS) ***

| Indicators | % |
|---|---|
| Distorted body perception (e.g. you feel/feel some part of your body is too fat, you think/think that losing weight will get rid of your problems). | 92.7 |
| Fear of weight gain | 81.0 |
| Dividing foods into «healthy» and «unhealthy» (orthorexia) | 80.5 |
| Stress-induced snacking episodes | 52.2 |
| Resorting to diets, starvation, exercise to burn calories | 47.3 |
| Obsessive thoughts about food, diets, calories, exercise | 42.4 |
| Calorie counting, strict food restrictions | 41.5 |
| Guilt over what you ate, punishing yourself. | 41.5 |
| Continuous weighing | 28.8 |
| Lack of control (eating large amounts of food in a short period of time) | 27.3 |
| Resorting to cleansing the stomach after eating with vomit or pills | 25.4 |
| The shame of eating in public | 24.4 |
| Following certain eating rituals (eating/ate when standing up, planning/planned your breakdown or overeating, sorting/sorted food by colour, not eating/didn't eat a certain food if you don't know/didn't know how many calories it has) | 17.6 |
| Constant cravings for certain foods, such as chocolate, fast food, etc. | 17.1 |
| Weight problems (your weight fluctuates/was fluctuating due to dieting; weight is lower than normal, but you continue/continued to lose weight) | 15.1 |
| Fear or refusal of meetings as there may be «unhealthy» food, etc. | 13.2 |
| No sense of satiety or hunger | 7.8 |

**Note.** * – the sum of answers exceeds 100 %, because the respondents were given an opportunity to choose several answer options.

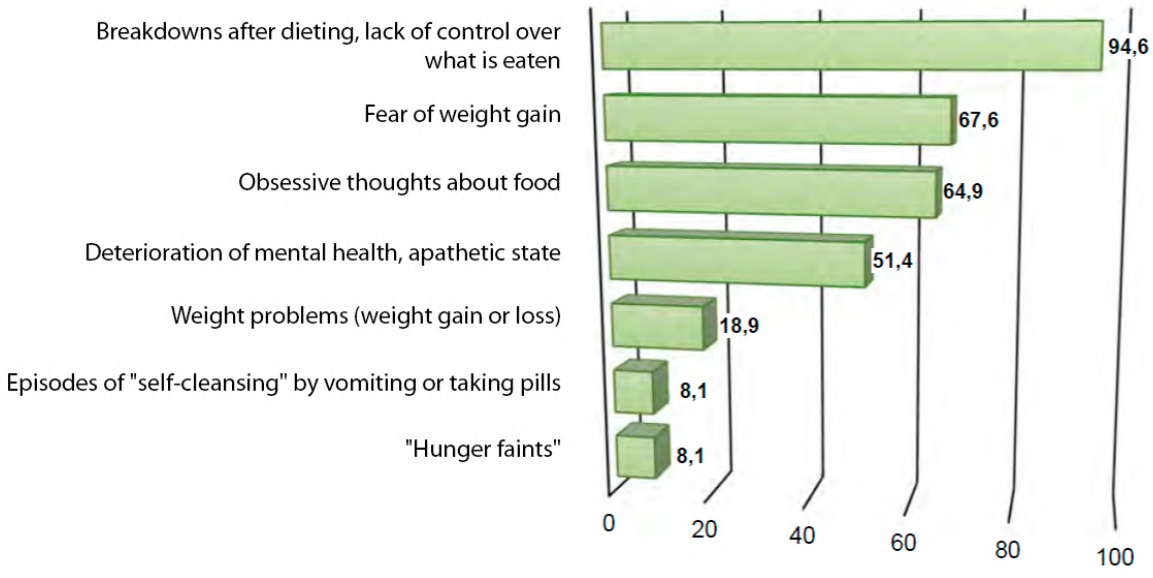


***FIG. 1.***
*Consequences of restricting eating behaviour in order to achieve an ideal body model (% of respondents): the sum of responses exceeds 100 % because respondents were given the opportunity to choose more than one answer option*

*that it only makes things worse and provokes the disease even more; especially since it is as if they romanticise it all, present it as something good, although there is total propaganda of ED»* [I8]; *«terrible communities, which should be banned, cause enormous harm to adolescent girls and young girls»* [I3]; *«such communities only exacerbate the problem..... like the crab in a bucket effect, when one wants to get out, others climb on top of him, and in the end no one gets out. ...In general, such groups should be banned, because it is very addictive»* [I6].

Such communities often publish photos of girls who are emaciated, extremely thin, with imbalanced proportions. Every third girl noted that she saw such photos quite often, and 30.2 % answered that they observed these photos every time they visited the community page.

We turned to the emotions that emerge in respondents when viewing such photos (Table 3). J. Berger and K.L. Milkman found that people are more likely to convey emotionally charged information that elicited either a very positive response or a very negative response [18]. The most common emotions experienced when viewing such content are sadness, outrage, aggression and nervousness.

Turning to the content published in communities about ED, the girls interviewed noted that they most often encountered photos of girls asking for feedback on their figure, encouraging weight loss and avoidance of food, and suggesting diet plans (Figure 2).

The influence of materials published in the communities was also confirmed by the interviewees: *«diseases are romanticised there: everyone who "sits" there only adds oil to the fire. You see these dead girls with thin legs and arms every day, it makes you feel bad because you want the same thing, but you can't do it. And it makes you hate yourself even more»* [I1]; *«full romanticisation of diseases, some jokes about ED, although not a funny topic at all. They post pictures of anorexic women and take it as an ideal figure, it's horrible»* [I3]; *«broadcasting a deliberately false image of the "correctness" of the figure or nutrition, it can destroy the psyche of a person in general»* [I7]; *«people decide for themselves whether to "sit" there or not. But personally, I don't understand it. I think it just makes it worse*

**TABLE 3**

**EMOTIONS EXPERIENCED WHEN SEEING PICTURES OF GIRLS WITH UNHEALTHY THINNESS***

| Emotions | Index | Rank |
|---|---|---|
| Sadness | 2.9945 | I |
| Exasperation | 2.6593 | II |
| Aggression | 2.2418 | III |
| Nervousness | 2.1703 | IV |
| Disgust | 1.8791 | V |
| Inspiration | 1.5549 | VI |
| Superiority complex | 1.5495 | VII |
| Envy | 1.4121 | VIII |
| Delight | 1.3297 | IX |

**Note.** * – respondents' evaluations were made on a 5-point scale, where 1 – never experienced an emotion; 5 – always experienced an emotion. The index is calculated in the range from 1 to 5. The higher the index value, the more often the girl experienced the emotion.

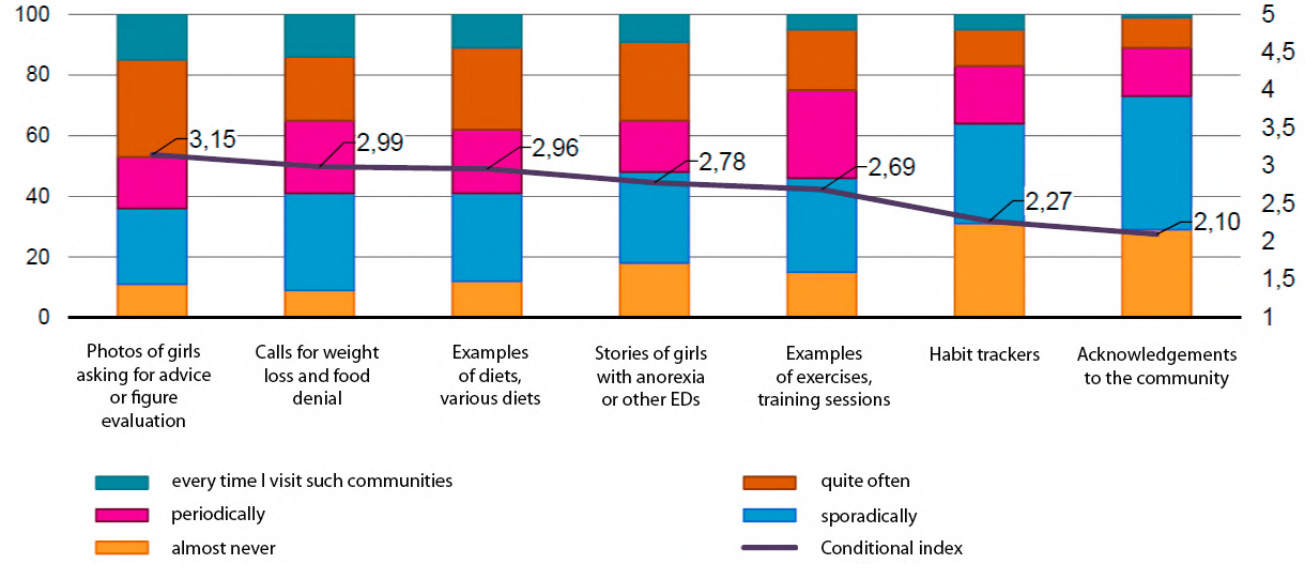


***FIG. 2.***
*Frequency and focus of content viewed in communities with unhealthy weight loss topics: the index was calculated in the range from 1 to 5; the higher the index value, the more often the girl encountered relevant content in the community*

*and provokes the disease even more.»* [I8]. We consider it important to emphasise that despite the negative attitude in general towards communities promoting unhealthy thinness, the interviewed girls are, nevertheless, well aware of the materials posted, which form an attitude towards the anorexic body as an ideal model and the practice of dietary restriction/refusal to eat as an effective tool for achieving the standard.

Another factor that has a significant influence as to the formation of girls' perceptions of their ideal body image and their eating behaviour is their immediate environment: classmates/groupmates (29.6 %), girlfriends/friends (12.7 %) and parental family (12.5 %). The interview participants confirmed the significant role of contact groups in the process of formation of ideas about the standard of corporeality: «*...not imposed, but as if it is unconsciously being digested by the brain. From some conversations in the environment, that is, nobody directly says that a woman should be so-and-so, although they constantly discuss full girls*» [I4]. Informants attributed a special influence to boys/youth/men's assessment of ideal female bodies: «*At school they laughed at overweight girls, friends, especially boys...*» [I4], «*...bullying at school, society's stereotypes, the opinion of some left-wing guys who say that "a woman should be this and that"...*» [I1]; «*...some men think it is their duty to write some girl a bad word about her figure*» [I3]; «*people can bully for being overweight, especially men, because a woman should always be perfect, and a man is a little prettier than a monkey* [I7] Male chauvinism becomes a significant factor in the formation of deviant eating behaviors. According to our online survey, 18.5 % of girls believe that a stereotype circulates in public opinion that when eating out, girls' portions should be small *(«a girl should eat little»)*, and each third shares the stereotype that it is necessary to give up eating out in favour of weight loss (*«to lose weight, all you have to do is stop eating»*).

The parental family also acts as an important agent in shaping the eating habits of the younger generation. In the gastronomic culture of some families, for example, one can observe the fascination of female mothers with dietary practices that are carried out without medical supervision. By forcing a child to finish a meal, parents encourage them to be guided not by how they feel, but by factors such as approval or permission to finish eating/leave the table. In our study, we invited girls to identify food attitudes formed in childhood that have a negative impact on overall health. Materials of the online survey reveal that the most widespread are such attitudes as rewarding with a sweet dessert after a full meal *(«you will get a chocolate bar only after you have eaten porridge»)* (65.9 %); watching TV or content on a laptop/tablet/phone during a meal (51.7 %); delaying a meal if one of the family members is late (20 %); finishing a meal only after all family members have eaten (18.5 %).

The materials of in-depth interviews also testify to the significant role of the parental family: *«personally, my mother and grandmother had a great influence on me: they made me always finish eating, even if I had already had enough, now it is as if I have no sense of satiety, I can eat endlessly»* [I4]; «*...family attitudes: when, for example, they forbid sweets, I want to eat them even more...*» [I3]; «*and «Sovietesque»* [«Sovietesque» – related to, correlated with Soviet (in the sense of «Soviet man», «Soviet power», «Soviet system»)] *attitudes that parents can instil: «you will not leave the table until you eat everything», «I will give you a candy only after soup», and so on. And my grandmother always said: «why are you so thin, eat up!»* [I7].

In the course of in-depth interviews, girls paid attention to the fact that external influences can cause psychological and physical tension (stress/anxiety), the way out of which becomes an eating disorder: «*... constant stress, against the background of which eating of emotions appears... Because of school, some academic failures, because of failed relationships, because of family quarrels... Food for ED women is like a cure for all diseases*» [I1]; «*food is perceived as a sedative*» [I3]; «*it may be associated to chronic stress, when a person cannot cope with it, and ED may develop. After all, food is dopamine, and a person tries to drown out stress with this dopamine*» [I4]; «*... stress in various situations, which induces to eat emotions, or on the contrary - to refuse to eat*» [I6]; «*I think this disease* (ED) *"comes out" against the background of stress. I've heard this theory that when a person can't control something in his life, he starts to control himself, his food, his body... the focus shifts*» [I8]. The questionnaire also revealed that 46.8 % of girls "escape" from stress by eating more food, and 44.4 %, on the contrary, eat less, up to complete refusal to eat. In addition, the findings revealed that girls who experience stress almost every day have a confirmed diagnosis from the eating disorder spectrum (31 %). The data demonstrate that the external environment influences the mental and physical state of the body, shaping deviations in food intake practices and creating conditions for risky eating behavior.

## CONCLUSION

The results of the conducted study led to the following conclusions. Distorted body perception, fear of gaining weight, and orthorexia are the most common signs of eating disorders, according to girls. At the same time, the key social factors influencing their formation are stereotypes about the ideal model of the female body, as well as the close contact circle, including relatives and friends/acquaintances. In the era of digitalisation, social media, including social networks, have a special influence upon perceptions of beauty standards and ways of achieving it. The study results reveal that girls have a negative attitude towards them, but are nevertheless involved in interacting and production of content. To meet the standard, the interviewed girls use dieting practices, food restriction and calorie counting, with a significant proportion of respondents recognising their negative consequences (breakdowns after diets, fear of gaining weight again, obsessive thoughts about food, deterioration of mental health).

The body today becomes a construct embedded in the socio-cultural space [19], which leads to the emergence of a certain discourse: ideal body – eating behavior. Social media has the potential to be a significant resource in representing a healthy body and demonstrating healthy eating behaviours. We assume that positive effects may be observed with the widespread replication of eating disorder recovery communities and social media groups, where real people share their stories of «battles» with eating disorders. The expert opinion of specialists in the field of treatment of this kind of disorders is also important, who will provide advice and support in a dialogue mode using social media. Special attention should be paid to the family institution, which also needs professional counselling aimed at establishing and developing a culture of eating behaviour in both the adult members of the family and the younger generation.

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**Conflict of interest**

The authors declare the absence of apparent and potential conflicts of interest related to the publication of this article.

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## CARDIOLOGY

# THE ROLE OF L-ARGININE IN THE PATHOGENESIS OF ESSENTIAL ARTERIAL HYPERTENSION

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## ABSTRACT

*The role of arginine in the development of primary arterial hypertension continues to be clarified up to the present moment. During natural metabolic processes in cells, methylated forms of arginine are produced – symmetric (SDMA) and asymmetric (ADMA) dimethylarginine. ADMA is a nitric oxide synthase inhibitor and is now considered a well-established marker for endothelial dysfunction. SDMA is not a nitric oxide synthase inhibitor, but may indirectly reduce nitric oxide production through competitive interaction with cellular L-arginine.*
*Currently, arginine preparations are practically not used for the treatment of primary arterial hypertension. This was the rationale for the given scientific review. The article summarizes the information available in the literature (2018–2022) on the pathogenetic mechanisms of the relationship between arginine and the development of impaired vascular tone. We used PubMed and RSCI databases for our review. Using keywords, 1784 publications were found over the past 5 years. The final selection criteria were time frame and matching keywords. The review provides data on the increased ADMA concentrations in experimental hypertensive animals and individuals with essential hypertension. The role of arginine metabolites in the genesis of endothelial dysfunction and arterial hypertension and the prospects for the therapeutic use of this compound are discussed.*

***Key words:*** *primary arterial hypertension, arginine, symmetrical dimethylarginine, asymmetrical dimethylarginine, citrulline, nitric oxide, nitric oxide synthase, comorbidity*

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# L-АРГИНИН И АРТЕРИАЛЬНАЯ ГИПЕРТЕНЗИЯ: КЛИНИКО-ПАТОГЕНЕТИЧЕСКИЕ ВЗАИМОСВЯЗИ И КОМОРБИДНОСТЬ

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## РЕЗЮМЕ

*Роль аргинина в развитии первичной артериальной гипертензии до настоящего времени продолжает уточняться. Во время естественных обменных процессов в клетках образуются метилированные формы аргинина – симметричный (SDMA, symmetric dimethylarginine) и асимметричный (ADMA, asymmetric dimethylarginine) диметиларгинин. ADMA является ингибитором синтазы окиси азота и в настоящее время рассматривается в качестве общепризнанного маркера эндотелиальной дисфункции. SDMA не является ингибитором синтазы окиси азота, однако может косвенно снижать продукцию окиси азота посредством конкурентного взаимодействия с клеточным L-аргинином.*

*В настоящее время препараты аргинина практически не используются для лечения первичной артериальной гипертензии. Это явилось обоснованием данного научного обзора. Статья обобщает имеющуюся в литературе информацию (2018–2022 гг.), посвящённую патогенетическим механизмам взаимосвязи аргинина с развитием нарушения сосудистого тонуса. Использованы базы данных PubMed, РИНЦ. По ключевым словам найдены 1784 публикации за последние 5 лет. Критериями окончательного отбора были временные рамки и совпадение ключевых слов. В обзоре приведены данные о повышении концентрации ADMA у экспериментальных гипертензивных животных и лиц с эссенциальной гипертензией; обсуждена роль метаболитов аргинина в генезе эндотелиальной дисфункции и артериальной гипертензии и перспективы терапевтического использования данного соединения.*

***Ключевые слова:*** *первичная артериальная гипертензия, аргинин, симметричный диметиларгинин, асимметричный диметиларгинин, цитруллин, оксид азота, синтаза оксида азота, коморбидность*

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Essential hypertension is the most common non-communicable disease, occurring in more than 30–45 % of the population worldwide and in the Russian Federation. The prevalence of this pathology determines the risk of coronary heart disease, myocardial infarctions, strokes, dementia, chronic kidney disease [1]. This determines the constant attention of scientists and clinicians to the pathogenesis of high blood pressure (BP). In our country, the term «hypertensive heart disease», proposed in 1948, is traditionally used. G.F. Lang, to define a disease whose main symptom is an increase in BP that is not associated with pathology leading to secondary hypertension. Overseas, the terms «essential hypertension» and/or «arterial hypertension» (AH) are synonymous with this diagnosis.

The founders of the pathogenetic links in the formation and progression of hypertension are G.F. Lang and A.L. Myasnikov, who described the neurogenic theory of hypertension. These days it is a recognised section of pathogenesis related to activation of the sympathoadrenal system. Since this mechanism of BP increase is realised mainly through alpha- and beta-adrenoreceptors, beta-adrenoreceptor blockers are widely used in the clinic [2]. Alpha-blockers are used less frequently, mainly in therapy-resistant hypertension and in individuals with benign prostatic hyperplasia.

Essential hypertension has been studied from various perspectives, ranging from genetics [3, 4], physiology [5], and in recent years microbiota [6-8] as previously underestimated areas of knowledge contributing to the identification of the aetiology of the disease. Dysfunction of the endothelium with predominant production of vasoconstrictor substances with a simultaneous decrease in the production of bradykinin, nitric oxide, prostacyclin and other compounds that reduce vascular tone plays a crucial role in the formation of hypertension.

Each direction individually has made unique contributions to the understanding of the different mechanisms of BP regulation. However, metabolic dysfunction as one of the links in the pathogenesis of primary arterial hypertension (PAH) is poorly understood. The amino acid L-arginine serves as the main substrate for nitric oxide (NO) production in blood vessels. NO has been called the «molecule of the twentieth century», as the use of drugs based on this molecule significantly improved the prognosis of patients not only with cardiovascular diseases, but also with other pathologies. L-arginine has previously been evidenced to reduce systemic blood pressure in some forms of experimental hypertension, but the comorbid pathology is poorly understood. Previous studies, although not uniform, have revealed positive results of the effects of L-arginine supplementation on endothelial function. As yet, however, they have not found widespread use in clinical practice. The above was the rationale for this scientific review.

## THE AIM

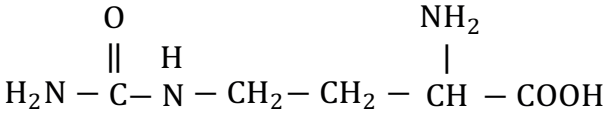
Study of the arginine role in the regulation of endothelial function and vascular tone.

This review summarises the information available in the literature (2018–2022) addressing the pathogenetic mechanisms of arginine's relationship with the development of vascular tone disorders. We used PubMed and RSCI databases for our review. The key words for the search were «primary arterial hypertension», «arginine», 'symmetrical dimethylarginine', 'asymmetrical dimethylarginine', 'citrulline', 'nitric oxide', and 'nitric oxide synthase'. 1,784 publications were found by keywords, mostly in the last 5 years. The final selection criteria were time frame and matching keywords.

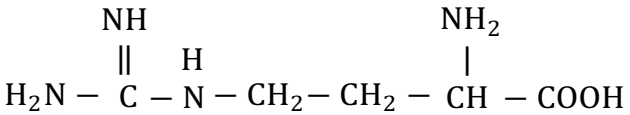
## 1. ROLE OF ARGININE IN PHYSIOLOGICAL CONDITIONS

In 1998, American scientists Louis J. Ignarro, Ferid Murad and Robert Francis Furchgott were awarded the Nobel Prize in Physiology and Medicine for their discovery of the role of nitric oxide as a signalling molecule in the regulation of the cardiovascular system. With the discovery of the NO role, new opportunities for the treatment of cardiovascular diseases have emerged.

L-arginine, hereinafter referred to as arginine, is a semisubstitutable or conditionally essential amino acid since it can be synthesised by healthy individuals [9]. The name comes from the Greek word ἄργυρος (silver), the typical colour of arginine nitrate crystals. Its chemical formula is 2-amino-5-guanidinopentanoic acid. In the body, arginine is synthesized from L-citruline. The L-citruline molecule is converted by argininosuccinate synthase enzymes into an intermediate product, argininosuccinate, which is cleaved by argininosuccinate lyase to arginine and fumarate. Through fumarate, the arginine conversion cycle and NO formation are linked to the tricarboxylic acid cycle. Arginine is used in cells to synthesise not only NO, but also proteins, urea, creatinine, polyamines, proline, and glutamate [10]. Arginine is involved in a number of biological processes, being the basis of many reactions for the synthesis of other amino acids, as well as a substrate for two enzymes: nitric oxide synthase (NOS) and arginase, which are essential for the formation of NO and urea, respectively.



**Citrulline formula**



**Arginine formula**

The definition of «nitric oxide» refers to the reduced form of NO with a half-life from 2 to 30 s [11, 12]. Its stable final metabolites are nitrite ($NO_2^-$) and nitrate ($NO_3^-$). Total indicator (nitrite and nitrate) is a product of NO, which is an indirect marker of nitric oxide concentration in the body. In body fluids, including plasma, most nitrite is converted to nitrate [13].

The nitric oxide synthase family includes endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). These NOS isoforms catalyse the conversion of arginine and L-homoarginine to NO, one of the most potent physiological vasodilators and inhibitors of platelet aggregation [14]. NO and other endothelial substances including prostacyclin (vasodilator) and endothelin (vasoconstrictor) have been revealed to play important roles in cardiovascular physiology and pathology [15]. Altered endothelial NO homeostasis as a result of endothelial dysfunction has been revealed to lead to cardiovascular diseases [16].

NO is synthesised from arginine by the enzyme NOS in a reaction involving electron transfer from nicotine amide adenine dinucleotide phosphate (NADP) – via flavinadenine dinucleotide and flavinmononucleotide in the C-terminal reductase domain – to heme in the N-terminal oxygenase domain, where the substrate arginine is oxidised to citrulline and NO [17]. The formation of NO occurs in two steps. First, NOS hydroxylates arginine to Nω-hydroxy-arginine (which remains largely bound to the enzyme). NOS then oxidises Nω-hydroxyarginine to citrulline and NO [18]. Under normal conditions, NOS catalyses the conversion of arginine, $O_2$ and NADPH electrons into NO and citrulline. However, in the presence of pathological conditions such as atherosclerosis and diabetes mellitus, NOS function is altered and the enzyme catalyses the reduction of $O_2$ to superoxide ($O_2^-$), a phenomenon commonly referred to as «NOS uncoupling» [19, 20].

## 2. THE ROLE OF ARGININE IN PATHOLOGY

One of the main mechanisms for the development of PAH is endothelial dysfunction [21-23]. In addition to PAH, it plays a role in the development of other diseases, including diabetes, atherosclerosis, and others. More importantly, many authors have revealed that the systemic manifestations observed in COVID-19 can be explained by endothelial dysfunction [24-26]. Actually, alterations in endothelial function have been associated with hypertension, diabetes mellitus, thromboembolism and renal failure, which have been observed to varying degrees in COVID-19 patients [27-30]. H.M. Al-Kuraishy, et al. [31] revealed that due to antiviral and immunomodulatory effects, L-arginine and released NO have interrelated effects against SARS-CoV-2 infection.

Over recent years, there has been growing interest in the potential therapeutic effects of arginine supplementation, especially in cardiovascular disease. Disruption of NO synthesis is considered a major sign of endothelial dysfunction [32]. Concurrently, some authors have revealed that arginine intake in healthy subjects does not significantly increase NO production [33]. For instance, daily administration of citrulline or arginine during 8 days to 15 well-trained swimmers had no effect on serum NO concentration, nor did it improve their performance at 100 and 200 m [34].

That said, other studies reveal the positive effects of arginine supplementation in healthy individuals. For example, arginine supplements have been tested on athletes because vascular dilation promotes oxygen perfusion during exercise, enhancing muscle strength and recovery [35]. These studies have produced conflicting results, sometimes finding no effect of arginine supplementation over physical performance and sometimes finding a significant improvement in exercise tolerance [36, 37].

The paradox of arginine is that although its intracellular physiological concentrations are only a few hundred micromoles per litre, thus exceeding the Km of eNOS, acute administration of exogenous arginine further increases NO production [38]. One mechanism that may help explain the arginine paradox is related to the discovery of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor [39].

Symmetric dimethylarginine (SDMA) is not a NOS inhibitor, but can indirectly reduce NO production through competitive interaction with cellular L-arginine [40]. SDMA is a methylated arginine amino acid. SDMA, together with its biologically active structural isomer ADMA, is formed as a consequence of intranuclear methylation of L-arginine residues of various regulatory proteins and is released into the cytoplasm after proteolysis. SDMA is excreted by the kidneys, while ADMA is largely metabolized [41].

Increased ADMA concentrations have been revealed in hypertension in experimental animals [42, 43]. An increase in ADMA concentration was recorded with PAH. Thus, in children aged 12–17 years, this index was observed to increase to 0.640 ± 0.017 μmol/l, which was statistically significantly ($p < 0.01$) higher than the level of the control group – 0.27 ± 0.02 μmol/l [44]. In paediatric PAH, ADMA levels were directly correlated with beta-salusin, but no relationship between beta-salusin and SDMA was revealed [45]. A cross-sectional study involving school children aged 6–9 years from the Eastern Cape Province of South Africa revealed a direct correlation between obesity, hypertension and ADMA [46].

V.I. Podzolkov et al. revealed a statistically significant increase in ADMA concentration in patients with essential arterial hypertension compared to physiological norm. Moreover, this increase was greater in the group with uncontrolled AH (UAH) compared to controlled AH (CAH). In intergroup analysis among patients with UAH, there was a significant positive correlation between ADMA concentration and creatinine level ($r = 0.615$; $p < 0.05$), and a statistically significant

negative correlation between ADMA level and renal filtration function assessed using the glomerular filtration rate ($r$ = -0.444; $p < 0.05$). With increasing levels of ADMA in serum, a statistically significant decrease in glomerular filtration rate was observed ($p < 0.05$). Also, a statistically significant positive correlation between ADMA content and progression of brachiocephalic artery stenosis was revealed in the group of patients with UAH ($r = 0.5$; $p < 0.05$). The authors believe that statistically significant correlation between ADMA level in patients with UAH and parameters of renal function decline and progression of brachiocephalic artery stenosis indicates the potential use of arginine as a marker of target organ damage and prognosis of the disease course [47].

Considering its similar structure to arginine, ADMA is a direct competitor for NOS binding. More importantly, both ADMA and arginine are transported into the cell via a highly affinity Na+-independent carrier of basic amino acids [48], and therefore also compete with each other at this level. Since ADMA competes with arginine for NOS and cellular transport, NO bioavailability depends on the balance between the both.

Arginine administration can balance the arginine/ADMA ratio, restoring NO production. The increased availability of arginine as a result of ingestion, in other words, competes with ADMA in binding eNOS. This interesting mechanism sheds light into the efficacy of increased arginine availability, suggesting its further therapeutic potential [49].

## 3. DISRUPTION OF NO PRODUCTION AS A MECHANISM OF ENDOTHELIAL DYSFUNCTION AND ARGININE INTERVENTION

The ability of the endothelium as a regulator of vascular homeostasis is largely dependent on NO production, making insufficiency of endothelial vasodilators a major sign of endothelial dysfunction. The impaired endothelial availability of NO in the vascular network may be associated with decreased NO synthesis or, indirectly, with increased production of reactive oxygen intermediates (ROIs), which inactivates the NO source [50]. In addition to counteracting oxidative stress, stimulation of NO synthesis represents an alternative and potentially effective approach, for example by providing additional substrates to NO synthase. Arginine supplementation theoretically fulfils these needs and therefore they have been tested in many cardiovascular diseases as a potential therapeutic strategy [51]. Studies examining the use of arginine on humans, however, have often been controversial. Actually, in healthy individuals as well as in patients with cardiovascular disease, plasma arginine levels range from ~45 to ~100 μmol/L [52], which is significantly higher than the eNOS Km of 2.9 μmol/L. Endocrine mechanisms may also contribute to arginine-induced vasodilation. In fact, arginine stimulates the release of insulin and glucagon by pancreatic islets of Langerhans [53].

Numerous data indicate that endothelial dysfunction is common in many diseases. A.A. Khan et al. revealed it in atrial fibrillation and associated a high risk of complications with endothelial dysfunction [54]. Endothelial dysfunction and C-reactive protein predict the incidence of heart failure in patients with arterial hypertension [55]. Endothelial dysfunction is also associated with age-related decline in cognitive and physical function [56], as well as with the pathogenesis of stroke [57], diabetes mellitus [58], erectile dysfunction [59] and heart failure [60].

Clinical studies examining the effects of arginine against endothelial dysfunction caused by aging have yielded conflicting results. Acute intravenous infusion of arginine (1 g/min for 30 min) had no effect on endothelial-dependent vasodilation in healthy elderly subjects [61]. However, intravenous arginine administration caused a significant increase in renal plasma flow, glomerular filtration rate, natriuresis and potassauresis in young but not in elderly hypertensive patients [62].

## 4. ARGININE INTAKE IN HYPERTENSION

Most studies in animal models have confirmed the beneficial effects of citrulline and arginine supplementation in elevated BP. The arginase pathway is responsible for the catabolism of 76–85 % and 81–96 % of arginine in the extraintestinal tissues of pigs and rats, respectively. Arginine supplementation (315 and 630 mg Arg/(kg body weight per day) for 91 days) had no adverse effects on male and female pigs. Similarly, no safety concerns were observed in male or female rats supplemented with 1.8 and 3.6 g arginine/(kg body weight per day) for at least 91 days. Intravenous administration of Arg-HCl to pregnant ewes at doses of 81 and 180 mg Arg/(kg body weight per day) is safe for at least 82 and 40 days, respectively. Animals receiving a normal diet can tolerate large amounts of Arg (up to 630 mg Arg/(kg body weight per day) for pigs or 3.6 g Arg/(kg body weight per day) for rats) well for 91 days, equivalent to 573 mg Arg/(kg body weight per day) for humans. Collectively, these results may help in studies to determine the safety of long-term oral administration of Arg in humans [63].

Brazilian authors have assessed whether endothelial function of vertebral arteries (VA) is impaired in men with hypertension. In 13 men with arterial hypertension (46 ± 3 years) and 8 men from the control group of the same age (46 ± 4 years), BP (photoplethysmography), blood flow in the vertebral (VA) and common carotid artery (CCA) was determined by duplex ultrasound. Results were recorded at rest and within 30 min after intravenous injection of L-arginine (30 g) or isotonic solution. The control group and hypertensive patients demonstrated similar blood flow at rest (601 ± 30 ml/min vs. 570 ± 43 ml/min in controls; $p = 0.529$) and blood flow in VA (119 ± 11 ml/min vs. 112 ± 9 ml/min in controls; $p = 0.878$). During L-arginine administration, blood

flow in the CCA increased equally between groups (12 ± 3 % in the group with AH and 13 ± 2 % in the control group; $p$ = 0.920). In contrast, increased blood flow in VA was absent in subjects with hypertension (0.8 ± 3 % compared with controls, 16 ± 4 %; $p$ = 0.015) without significant change in BP. Flows in both CCA and VA returned to near resting values within 30 minutes after infusion, and four patients with hypertension and three of the control group had no significant effect on blood flow in VA or CCA. The results demonstrate endothelial dysfunction in the posterior cerebral circulation in middle-aged men with arterial hypertension [64].

Iranian authors studied the effect of L-arginine supplementation over BP by conducting a systematic review and meta-analysis of dose-effect relationships in randomised placebo-controlled clinical trials (RCTs). They searched online databases for relevant keywords up to April 2021 to identify RCTs using oral L-arginine supplementation to measure systolic BP (SBP) and diastolic BP (DBP) in adults. Inclusion criteria were adult participants and duration of intervention ≥ 4 days. Exclusion criteria were L-arginine infusions and emergency interventions. A random effects model was used to estimate the weighted mean difference (WMD) and 95% confidence interval (95% CI). 22 RCTs were included in this meta-analysis. Pooled analysis demonstrated significant reductions in SBP (WMD = -6.40 mmHg; 95% CI: -8.74; -4.05; $p$ < 0.001) and DBP (WMD = -2.64 mmHg; 95% CI: -3.94; -1.40; $p$ < 0.001) after L-arginine supplementation. Subgroup analyses showed significant reductions in SBP and DBP regardless of baseline BP category (normotensive, hypertensive), study duration (≤ 24 days, > 24 days), sex (female, male), health status (healthy, unhealthy), and body mass index (normal, overweight, obese). No significant changes were observed at doses > 9 g/day, trial duration > 24 days, or in obese subjects. L-arginine supplementation also reduces DBP more effectively in women than in men. Moreover, meta-regression analysis of DBP demonstrated a significant association between the dose of L-arginine intake and DBP changes ($p$ = 0.020). In non-linear dose-response analysis, the effective dose of L-arginine was found to be ≥ 4 g/day for SBP ($p$ = 0.034) regardless of study duration. Overall, the authors believe that L-arginine supplementation may be effective in reducing BP [65].

Chinese authors have examined the effects of traffic-related air pollution over BP, cardiovascular disease, and mortality. They aimed to assess the potential efficacy of L-arginine supplementation in mitigating adverse cardiovascular effects in adults with elevated BP while walking outdoors under conditions of vehicular air pollution using a randomised, double-blind, placebo-controlled trial. A total of 118 adults with elevated BP were enrolled and randomly assigned to either a placebo group or an intervention group with L-arginine supplementation at a dose of 9 g/day for 2 weeks. On day 14, participants from the two groups walked in pairs along the carriageway for 2 hours. Resting BP, L-arginine nitric oxide metabolites, and inflammatory biomarkers were measured before, during, and after a 2-hour exposure, and BP measurement and Holter monitoring were performed during a 2-hour outdoor walk. Participants in the main group had significantly increased plasma L-arginine levels compared to the placebo group after supplementation. Both groups were exposed to the same traffic-related air pollutants. However, participants in the main group revealed a significant decrease of 5.3 mmHg (95% CI: -9.9; -0.7) in resting SBP, 4.3 mmHg – in resting DBP, and 4.6 mmHg (95% CI: -7.9; -1.3) in resting mean blood pressure (mBP) at 30 minutes after a 2-hour outdoor walk compared with the placebo group. There were also significant reductions in ambulatory SBP, DBP, and mBP (7.5–9.9 mmHg, 5.3–7.6 mmHg, and 4.7–7.9 mmHg, respectively) during walking in the main group compared with the placebo group. No significant changes in ST segment levels, L-arginine (NO) metabolites and inflammatory biomarkers were revealed, and no statistically significant associations were observed between specific traffic-related air pollutants and measures of cardiovascular health. The study reveals that oral L-arginine supplementation was safe and well tolerated, and could improve BP levels in adults with elevated BP while walking in the air, even when it was polluted due to traffic [66]. Other authors also reveal a favourable effect of arginine supplementation on the course of PAH [67-69].

## CONCLUSION

In summary, the amino acid arginine is a molecule involved in BP regulation. Asymmetric dimethylarginine and its structural isomer, symmetric dimethylarginine, are predictors of the development of a complicated course of cardiovascular disease. Previous studies conducted in relation to the therapeutic use of arginine are contradictory, making it difficult to put the results into practice.

Overall, the literature data recommend arginine supplementation for cardiovascular disease, especially to prevent the development of hypertension and atherosclerosis. One of the limitations of arginine supplementation remains the selection of the optimal target group. With respect to these concerns, we believe that ADMA levels can be very useful in selecting a target population, and patients with an elevated ADMA/arginine ratio are probably the most appropriate group for whom arginine supplementation may indeed be effective. Another limitation to the use of arginine concerns its dosage. Indeed, the available evidence offers a range of different doses – sometimes effective, sometimes not. Unfortunately, much of the evidence about the effects of arginine with regard to hypertension comes from small clinical trials, and despite the promising efficacy, further, especially large, randomised and controlled trials are needed.

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# ASSOCIATION OF FoxP3+ T REGULATORY LYMPHOCYTES WITH EPICARDIAL ADIPOSE TISSUE THICKNESS IN PATIENTS WITH CORONARY HEART DISEASE

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## ABSTRACT

***Background.*** *Increase of the epicardial adipose tissue (EAT) thickness is associated with development of inflammation and cardiovascular complications, however, there is no data on the relationship between EAT thickening and the number of immunosuppressive regulatory T lymphocytes.*

***The aim.*** *To study the number of circulating T regulatory lymphocytes and nuclear translocation of the FoxP3 transcription factor in patients with stable coronary heart disease (CHD) depending on the epicardial adipose tissue thickness.*

***Materials and methods.*** *We examined 30 patients with chronic stable CHD. The EAT thickness was measured by echocardiography. Patients were divided into groups depending on the presence and absence of EAT thickening above 5 mm (groups 1 and 2, respectively). Imaging flow cytometry was used to determine the number of T regulatory lymphocytes and the level of FoxP3 nuclear translocation. The concentration of cytokines and high sensitivity C-reactive protein (hsCRP) was determined using enzyme-linked immunosorbent assay in blood serum.*

***Results.*** *In group 2, there was an increase in low-density lipoprotein cholesterol concentration (p = 0.043), ratio of low-density lipoprotein cholesterol to high-density lipoprotein cholesterol (p = 0.017) and the concentration of hsCRP (p = 0.044) and IL-1β (p = 0.005), a decrease in the number and relative count of T regulatory lymphocytes (p = 0.020 and p = 0.026, respectively), as well as the number of cells with FoxP3 nuclear translocation (p = 0.018) compared to group 1. According to multiple logistic regression, the concentration of hsCRP, IL-1β and T regulatory lymphocytes relative count in total were the predictors of EAT thickening (accuracy 80 %; sensitivity 75 %; specificity 84.6 %; AUC = 0.89).*

***Conclusion.*** *Thickening of epicardial adipose tissue in patients with coronary heart disease is associated with a decrease in the number of T regulatory lymphocytes and FoxP3 nuclear translocation in them in presence of comparable anthropometric parameters of obesity and the severity of coronary atherosclerosis.*

***Key words:*** *epicardial adipose tissue, T regulatory lymphocytes, FoxP3, interleukin 1β, lipids, imaging flow cytometry, atherosclerosis*

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# ВЗАИМОСВЯЗЬ СОДЕРЖАНИЯ FoxP3+ Т-РЕГУЛЯТОРНЫХ ЛИМФОЦИТОВ И ТОЛЩИНЫ ЭПИКАРДИАЛЬНОЙ ЖИРОВОЙ ТКАНИ У ПАЦИЕНТОВ С ИШЕМИЧЕСКОЙ БОЛЕЗНЬЮ СЕРДЦА

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## РЕЗЮМЕ

***Обоснование.*** *Увеличение толщины эпикардиальной жировой ткани (ЭЖТ) ассоциируется с развитием воспаления и сердечно-сосудистых осложнений, однако данные о взаимосвязи между утолщением ЭЖТ и количеством регуляторных Т-лимфоцитов отсутствуют.*

***Целью исследования*** *являлось изучение содержания циркулирующих Т-регуляторных лимфоцитов и ядерной транслокации фактора FoxP3 у пациентов со стабильной ишемической болезнью сердца (ИБС) в зависимости от толщины эпикардиальной жировой ткани.*

***Методы.*** *Обследовано 30 пациентов с хронической стабильной ИБС. Толщину ЭЖТ измеряли методом эхокардиографии. Пациенты были разделены на группы в зависимости от отсутствия и наличия утолщения ЭЖТ более 5 мм (группы 1 и 2 соответственно). Методом проточной цитометрии с визуализацией определяли содержание Т-регуляторных лимфоцитов и уровень ядерной транслокации FoxP3. Методом иммуноферментного анализа в сыворотке крови определяли содержание цитокинов и высокочувствительного С-реактивного белка (вчСРБ).*

***Результаты.*** *В группе 2 выявлено увеличение содержания холестрола липопротеинов низкой плотности ($p = 0,043$), соотношения холестерола липопротеинов низкой плотности к холестеролу липопротеинов высокой плотности ($p = 0,017$) и концентрации вчСРБ ($p = 0,044$) и IL-1β ($p = 0,005$) и снижение относительного и абсолютного содержания Т-регуляторных лимфоцитов ($p = 0,020$ и $p = 0,026$ соответственно), а также количества клеток с ядерной транслокацией FoxP3 ($p = 0,018$) по сравнению с группой 1. По данным множественной логистической регрессии концентрации вчСРБ, IL-1β и доля Т-регуляторных лимфоцитов в совокупности являлись предикторами наличия утолщения ЭЖТ.*

***Заключение.*** *Утолщение эпикардиальной жировой ткани у пациентов с ИБС ассоциируется со снижением содержания Т-регуляторных лимфоцитов в крови и ядерной транслокации фактора FoxP3 в них при сопоставимых антропометрических параметрах ожирения и выраженности коронарного атеросклероза.*

***Ключевые слова:*** *эпикардиальная жировая ткань, Т-регуляторные лимфоциты, FoxP3, интерлейкин-1β, липиды, проточная цитометрия с визуализацией, атеросклероз*

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## BACKGROUND

Epicardial adipose tissue (EAT) is a unique fat depot localised between the myocardium and the visceral layer of the epicardium. EAT is closely related to cardiac tissue both anatomically and functionally and contributes to the pathogenesis of a number of cardiovascular diseases such as coronary heart disease (CHD), atrial fibrillation and heart failure [1].

Increased EAT thickness determined by echocardiography is a reflection of ectopic fat deposition, which is associated with increased cardiovascular risk [2, 3].

It is evidenced that the content of immune cells in EAT is the highest compared to other fat depots and is about 20 % of the total number of cells [4]. EAT in patients with CHD is characterised by marked inflammation associated with infiltration by macrophages with predominantly proinflammatory M1 phenotype, mast cells and cytotoxic T-lymphocytes, and the severity of inflammation in EAT, as a rule, exceeds that not only in subcutaneous adipose tissue (SAT), but also in any other visceral fat depot, and the thickness of EAT directly correlates with the severity of inflammation in it [1].

Regulatory T lymphocytes (Treg) represent a distinct and important subpopulation of T lymphocytes that provides protection against the development of autoimmune reactions and excessive immune response. The $CD4^+CD25^{hi}FoxP3^+$ Treg subpopulation, which in norm is 5–10 % of all $CD4^+$ T-cells in peripheral blood and lymphoid organs, is the most characterized. Treg cell deficiency is associated with signs of subclinical inflammation in patients at high and very high cardiovascular risk and increased high-sensitivity C-reactive protein (hsCRP) [5]. Treg lymphocytes exert immunosuppressive function both by contact, through direct interreceptor interaction, and by secretion of anti-inflammatory cytokines. The key cytokines of Treg lymphocytes are interleukin (IL) 10 and transforming growth factor (TGF) β [6], while IL-1β suppresses their activity [7]. The state of lipid metabolism, including the activity of proprotein convertase subtilisin-kexin type 9 (PCSK9), has also been evidenced to be critical in maintaining Treg homeostasis [8, 9]. In the process of Treg lymphocyte activation, the expression of CD25 molecule may decrease, and cells with $CD4^+CD25^{lo}FoxP3^+$ phenotype appear. And the number of these cells increases with active autoimmune inflammation [10].

The suppressor activity of Treg is highly dependent on the stable expression and activity of the transcription factor FoxP3. When intracellular signalling is impaired, FoxP3 translocation to the nucleus is impaired, which is accompanied by Treg dysfunction [11]; however, post-translational regulation of FoxP3 activity has attracted the attention of researchers relatively recently, and many questions remain unexplored.

The existence of adipose tissue resident Treg lymphocytes has been evidenced to contribute to the maintenance of tissue insulin sensitivity by suppressing excessive inflammation. Visceral adipose tissue is most enriched with Treg lymphocytes, the content of which can reach 50 % of all $CD4^+$ T cells [12]. Fatty Tregs are characterised by a distinct repertoire of T cell receptors (TCR), produce more IL-10 and depend on PPAR-γ regulation, in contrast to Tregs of lymphoid origin [12].

Most of the information about the role of Treg in the regulation of visceral adipose tissue functional properties, however, has been derived from animal models, and studies in the human population are inconsistent. For example, when FoxP3 mRNA was assessed, it was revealed that visceral adipose tissue from obese patients contained less FoxP3 mRNA than visceral adipose tissue from normal weight individuals [13]. In contrast, in other studies, FoxP3 expression increased in obesity [14]. Considering that Treg in adipose tissue are most probably of thymic origin [15], it is of interest to investigate whether the changes occurring in EAT in patients with CHD are related to systemic immunoregulatory dysfunction and whether they are correlated with the content of circulating Treg lymphocytes.

**The aim of the present study** was to examine the content of $CD25^{hi}FoxP3^+CD4^+$ and $CD25^{lo}FoxP3^+CD4^+$ Treg lymphocytes and subcellular localisation of FoxP3 in them in patients with stable coronary heart disease depending on the thickness of epicardial adipose tissue.

## METHODS

An observational single-center, single-stage comparative study was conducted. The study included 30 patients (22 men and 8 women) aged 44 to 78 years with chronic stable CHD.

All procedures were performed in accordance with the World Medical Association Declaration of Helsinki «Ethical Principles for Conducting Scientific Medical Research Involving Human Subjects» as amended in 2004 and «Rules of Good Clinical Practice in the Russian Federation» approved by Order of the Ministry of Health of the Russian Federation No. 200н dated April 01, 2016. The study was approved by the local ethical committee of the Research Institute of Cardiology, Tomsk National Research Medical Centre of the Russian Academy of Sciences (Minutes No. 210 dated February 18, 2021). All individuals included in the study signed informed consent to participate.

The study included only those patients in whom coronary angiography and echocardiography procedures with EAT thickness determination were previously performed as indicated. Selective coronary angiography was performed using the Artis One angiography suite (Siemens Shenzhen Magnetic Resonance Ltd., China) and the Digitron-3NAC computer system (Siemens Shenzhen Magnetic Resonance Ltd., China). Gensini Score index [16] was used to assess the severity of coronary atherosclerosis.

EAT thickness was measured along the free wall of the right ventricle at the end of diastole [2, 17] at a point perpendicular to the direction of the ultrasound beam

with respect to the free wall of the right ventricle using the aortic annulus as an anatomical landmark. EAT thickness was determined based on the mean of echocardiographic data obtained in three consecutive cardiac cycles. EAT thickness (tEAT) greater than 5 mm was considered EAT thickening [18]. All patients were divided into two groups: group 1, without EAT thickening (tEAT ≤ 5 mm); group 2, with EAT thickening (tEAT > 5 mm).

Exclusion criteria comprised: acute atherosclerotic complications in the preceding 6 months; any acute inflammatory disease; presence of current or recurrent infectious disease; chronic kidney disease above C3b class; presence of oncological, haematological or autoimmune diseases; and refusal to participate in the study.

All patients underwent anthropometric measurements to assess overall obesity (body mass index (BMI) was calculated) and abdominal obesity (waist circumference was measured).

All patients were on regular drug therapy approaching optimal.

The material for the study was venous blood sampled in the morning on an empty stomach. Blood was sampled in tubes with heparin and tubes with clotting activator. The tubes with clotting activator were centrifuged at an acceleration of 1500 g. After centrifugation, serum was sampled into plastic microtubes, frozen at -40 °C and stored until further examination.

Mononuclear leukocytes were obtained from blood with heparin by density gradient centrifugation (Histopaque 1077, Sigma Aldrich, USA). To assess the content of $CD4^+CD25^{hi}FoxP3^+$ and $CD4^+CD25^{lo}FoxP3^+$ Treg lymphocytes, cells were stained with monoclonal antibodies to surface antigens (anti-CD4 conjugated with fluorochrome FITC; anti-CD25 conjugated with fluorochrome PE) (Becton Dickinson, USA), cells were fixed and permeabilized using a set of buffers for intracellular and intracellular staining (Becton Dickinson, USA). Cells were then stained with monoclonal antibodies to FoxP3 conjugated to fluorochrome AF647 (Becton Dickinson, USA), fixed and 7-aminoactinomycin D (7-AAD) was added to stain the nucleus. Cells were harvested on an Amnis FlowSight instrument (Luminex, USA) with 488 nm and 642 nm lasers in the INSPIRE program (Amnis Corporation, USA). For subcellular localization analysis of FoxP3, IDEAS 2.0 software (Amnis Corporation, USA) including the Nuclear Localization Wizard for cell image analysis was used. Results were expressed as % positive cells from the desired population. The absolute number of cells in the peripheral blood was calculated according to the data of the general blood analysis.

Concentrations of hsCRP, IL-10, IL-1 β (all kits – Vector-Best, Russia), TGF-β (Invitorgen kit, ThermoFisher Scientific, Austria), PCSK9 (R&D Systems kit, Bio-techne, USA) were assessed in serum by solid phase enzyme immunoassay.

Blood lipid spectrum (total cholesterol (TCL), triglycerides (TG), CL of high-density lipoprotein (CL-HDL), CL of low-density lipoprotein (CL-LDL) was studied (kits of CJSC «Diakon-DS», Russia). Blood glucose and glycated haemoglobin contents were estimated on Konelab 60i (ThermoFisher Scientific, USA) and Cobas 6000 C501 (Rosche, USA) automated analysers, respectively. The CL-LDL/CL-HDL ratio was calculated.

Statistical processing of the obtained data was performed using Statistica 10.0 software package (StatSoft Inc., USA). Results were presented as median and interquartile range (Me (Q1; Q3)). The Shapiro – Wilk criterion was used to test the hypothesis of normality of distribution of quantitative indicators. Since the distribution of quantitative indices differed from normal, the statistical significance of differences in quantitative indices in independent groups of patients was assessed using the Mann – Whitney U-criterion. The frequencies of occurrence in independent groups of patients were compared by Pearson's $\chi^2$ test or Fisher's exact test. Spearman's rank correlation coefficient($r_s$) was used to assess the relationship between the traits. Differences were considered statistically significant when the achieved level of $p < 0.05$. To reveal statistically significant factors affecting the increase in epicardial adipose tissue thickness, a multiple logistic regression model was constructed; to assess the quality of the model, a ROC-curve (Receiver Operator Characteristic) was constructed and the area under curve (AUC), accuracy, sensitivity and specificity of the model were calculated.

## RESULTS

The groups of patients with EAT thickness ≤ 5 mm and > 5 mm were comparable to each other in terms of sex, age, presence and duration of type 2 diabetes mellitus (DM) and arterial hypertension (AH), smoking status, anthropometric measures of obesity and severity of atherosclerosis (Table 1). The frequency of intakes of statins and the type of drug taken (atorvastatin / rozuvastatin) did not differ between patient subgroups (Table 1), but the dosages taken were higher in the group of patients with thicker EAT: 20 (20; 40) vs. 40 (40; 60) mg/day for atorvastatin ($p = 0.019$) and 20 (10; 20) vs. 30 (20; 40) mg/day for rosuvastatin ($p = 0.030$)).

When metabolic parameters were assessed, it turned out that patients with EAT thickness > 5 mm were characterised by higher CL-LDL concentrations and CL-LDL/CL-HDL ratio values (despite more intensive therapy with statins), and tended to have lower CL-HDL and higher PCSK9 concentrations. The glycemic indices in both groups were comparable (Table 2).

In patients with EAT > 5 mm, we revealed a decrease in the relative and absolute content of $CD25^{hi}FoxP3^+$ Treg lymphocytes, as well as a decrease in the absolute content of $CD25^{hi}FoxP3^+$ Treg lymphocytes with intracellular FoxP3 localization (Fig. 1). The content of $CD25^{lo}FoxP3^+$ Treg lymphocytes did not differ in the studied groups (Fig. 1).

The content of pro-inflammatory hsCRP and IL-1β was higher in patients with EAT thickness > 5 mm with comparable concentrations of TGF-β and IL-10, key Treg lymphocyte cytokines (Table 3).

In the general group of CHD patients, the content of $CD25^{hi}FoxP3^{+}$ Treg lymphocytes was negatively correlated with triglyceride concentration ($r_s$ = -0.345; $p$ = 0.034) and triglyceride/CL-HDL ratio ($r_s$ = -0.388; $p$ = 0.016), and the absolute content of $CD25^{hi}FoxP3^{+}$ Treg lymphocytes and $CD25^{hi}FoxP3^{+}$ Treg lymphocytes with intracellular localization of FoxP3 were negatively correlated with the concentration of IL-1β ($r_s$ = -0.374; $p$ = 0.038 and $r_s$ = -0.389; $p$ = 0.031, respectively). Only in the group of patients with EAT thickness ≤ 5 mm, we found an inverse relationship between PCSK9 content and absolute $CD25^{lo-}FoxP3^{+}$ Treg lymphocytes content ($r_s$ = -0.608; $p$ = 0.036). In patients with greater EAT thickness, this relationship was absent.

**TABLE 1**

**CHARACTERIZATION OF PATIENTS ACCORDING TO THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE**

| Indices | Patients with tEAT < 5 mm (*n* = 14) | Patients with tEAT > 5 mm (*n* = 16) | *p*-value |
|---|---|---|---|
| Sex: male/female | 10/4 | 12/4 | 0.999 |
| Age, years | 67 (59; 69) | 61 (58; 67) | 0.224 |
| AH history, *n* (%) | 14 (100) | 15 (93.8) | 0.998 |
| AH duration, years | 19.5 (15.0; 30.0) | 15.0 (12.0; 20.0) | 0.217 |
| type 2 DM history, *n* (%) | 7 (50) | 4 (25) | 0.477 |
| DM duration, years | 0.5 (0; 5) | 0 (0; 2) | 0.652 |
| BMI, $kg/m^2$ | 29.2 (25.7; 31.9) | 27.0 (24.3; 32.1) | 0.854 |
| Waist circumference, cm | 100 (96; 105) | 100.6 (93.0; 111.5) | 0.697 |
| tEAT, mm | 4.8 (3.9; 5.0) | 9.1 (7.8; 11.4) | < 0.001 |
| Gensini Score, points | 30.0 (17.5; 77.0) | 53.0 (38.8; 68.5) | 0.313 |
| Tobacco smoking, *n* (%) | 3 (21.4) | 8 (50.0) | 0.142 |
| Statin intake, *n* (%) | 14 (100) | 15 (93.8) | 0.998 |
| Atorvastatin, *n* (%) | 9 (64.3) | 8 (50.0) | 0.484 |
| Rosuvastatin, *n* (%) | 5 (35.7) | 7 (43.7) | 0.722 |

**TABLE 2**

**METABOLIC PARAMETERS OF PATIENTS DEPENDING ON THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE**

| Indices | Patients with tEAT < 5 mm (*n* = 14) | Patients with tEAT > 5 mm ( *n* = 16) | *p*-value |
|---|---|---|---|
| Fasting glucose, mM | 6.3 (5.3; 6.8) | 5.8 (5.2; 7.6) | 0.886 |
| Glycated hemoglobin, % | 6.1 (5.8; 8.3) | 6.2 (6.0; 6.6) | 0.701 |
| Total cholesterol, mM | 3.3 (2.9; 3.8) | 4.2 (3.0; 5.4) | 0.154 |
| Triglycerides, mM | 1.2 (0.8; 1.9) | 1.6 (1.1; 2.1) | 0.240 |
| CL-LDL, mM | 1.6 (1.1; 2.1) | 2.4 (1.5; 2.8) | 0.043 |
| CL-HDL, mM | 1.1 (1.0; 1.3) | 1.0 (0.8; 1.1) | 0.077 |
| CL-LDL/CL-HDL | 1.4 (0.9; 1.9) | 2.0 (1.6; 3.0) | 0.017 |
| PCSK9, ng/mL | 211.1 (180.7; 244.5) | 273.9 (199.3; 294.8) | 0.087 |

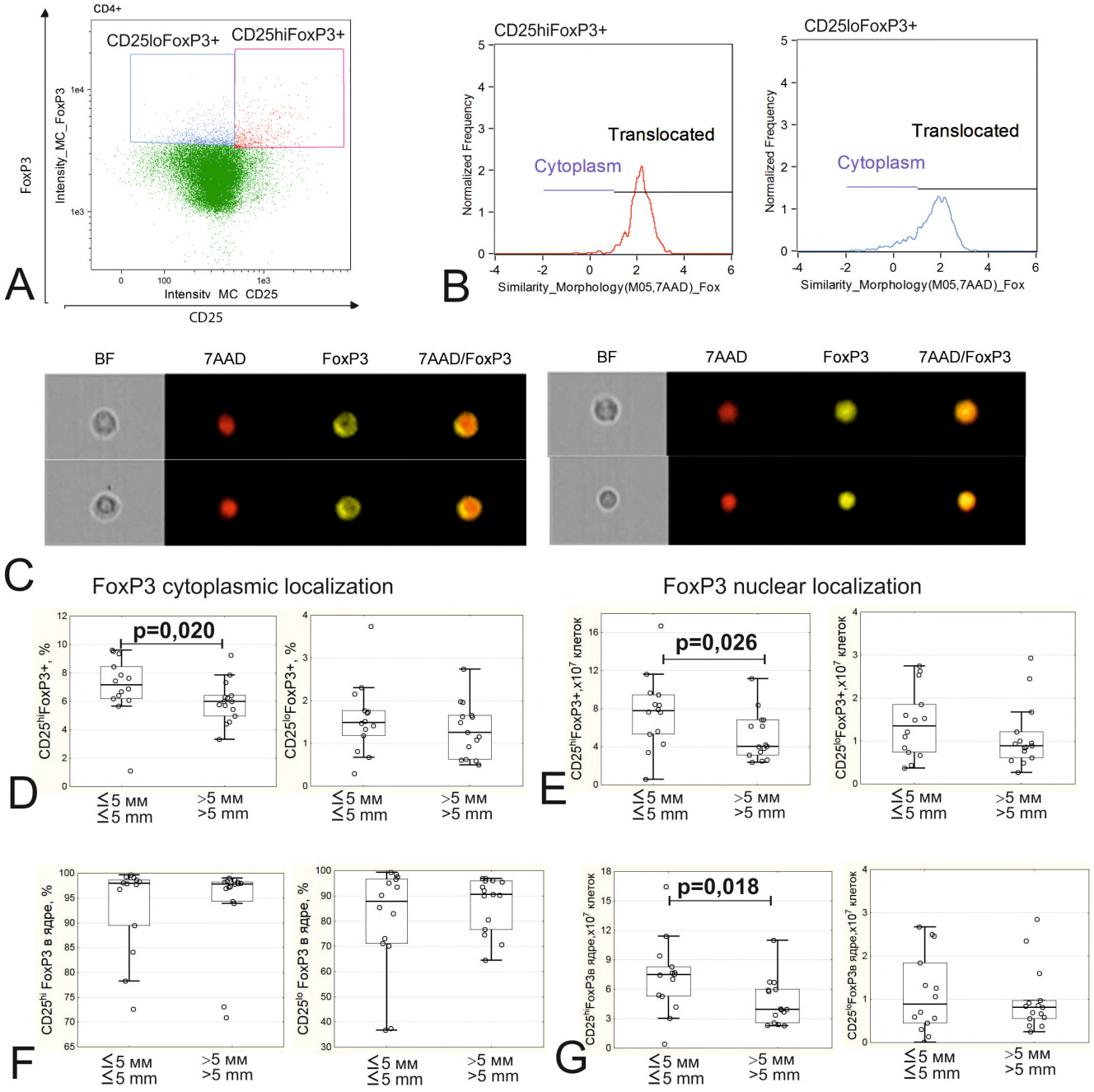


***FIG. 1.***
*The content of FoxP3+ T-regulatory lymphocytes and translocation of FoxP3 into the nucleus:* ***A*** *– an example of a dot diagram reflecting the content of CD25hiFoxP3+ and CD25loFoxP3+ cells among CD4+ lymphocytes;* ***B*** *– histograms reflecting the degree of translocation of FoxP3 into the nucleus;* ***C*** *– examples of cellular images obtained by flow cytometry with visualization with cytoplasmic and nuclear localization of FoxP3 (the nucleus is colored red, FoxP3 is colored yellow);* ***D*** *– the relative content of CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***E*** *– the absolute content of CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***F*** *– the proportion of cells with nuclear localization of FoxP3 among CD25hiFoxP3+ and CD25loFoxP3+ cells at different tEAT;* ***G*** *– the absolute content of CD25hiFoxP3+- and CD25loFoxP3+ cells with nuclear localization of FoxP3 at different tEAT*

**TABLE 3**

**CONTENT OF KEY CYTOKINES AND HIGH-SENSITIVITY C-REACTIVE PROTEIN IN PATIENTS ACCORDING TO THE THICKNESS OF EPICARDIAL ADIPOSE TISSUE**

| Indices | Patients with tEAT < 5 mm (*n* = 13) | Patients with tEAT > 5.4 mm (*n* = 13) | *p-value* |
|---|---|---|---|
| hsCRP, mg/L | 2.1 (1.0; 4.5) | 4.9 (3.1; 17.5) | 0.044 |
| TGF-β, ng/mL | 36.2 (29.1; 38.2) | 36.6 (32.4; 42.4) | 0.948 |
| IL-10, pg/mL | 1.9 (1.7; 3.2) | 2.3 (1.8; 2.6) | 0.545 |
| IL-1β, pg/mL | 0.7 (0.5; 0.9) | 1.1 (0.9; 1.4) | 0.005 |

**TABLE 4**

**ESTIMATES OF LOGISTIC REGRESSION MODEL COEFFICIENTS AND THEIR LEVELS OF STATISTICAL SIGNIFICANCE**

| Indices | Ratio estimation | *p value* |
|---|---|---|
| Absolute term | 0.228 | 0.929 |
| hsCRP, mg/L | –0.101 | 0.283 |
| IL-1β, pg/mL | –2.378 | 0.101 |
| $CD25^{hi}FoxP3^{+}$ Treg, % | 0.461 | 0.143 |

According to correlation analysis, EAT thickness in all patients with CHD was positively correlated with BMI ($r_s$ = 0.336; $p$ = 0.037), waist circumference ($r_s$ = 0.379; $p$ = 0.017), serum concentrations of hsCRP ($r_s$ = 0.400; $p$ = 0.019) and IL-1ß ($r_s$ = 0.444; $p$ = 0.008) and negatively – with a relative $CD25^{hi}FoxP3^{+}$ Treg lymphocytes content ($r_s$ = -0.353; $p$ = 0.032).

We constructed a multiple logistic regression model according to which the significant predictors of EAT thickening in patients were hsCRP concentration, IL-1β concentration, and relative content of $CD25^{hi}FoxP3^{+}$ T-regulatory lymphocytes (Table 4).

Although the individual contribution of each of the predictors in the model individually was not statistically significant, the level of statistical significance of the model as a whole was high ($p$ = 0.00403). The predictive performance of the model: accuracy 80.0 %, sensitivity 75.0 %, specificity 84.6 %, AUC = 0.891. The ROC curve of the model is shown in Figure 2. The threshold probability of different degrees of EAT thickening was 0.63.

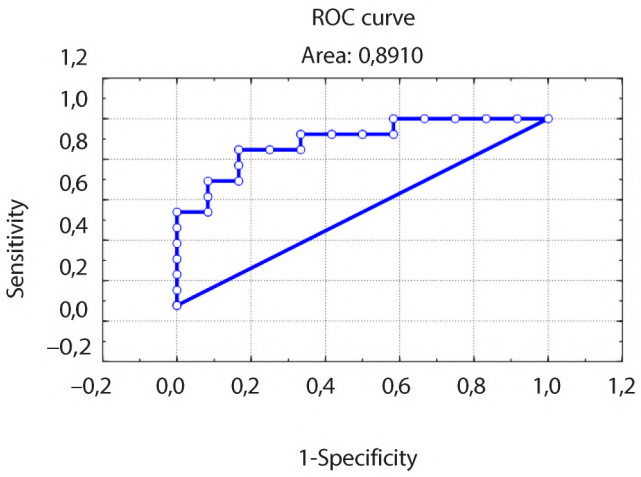


***FIG. 2.***
*ROC curve of the multiple logistic regression model for classifying patients into groups with and without thickening of epicardial adipose tissue thickness greater than 5 mm*

## DISCUSSION

Although increased EAT thickness is associated with increased local production and accumulation of proinflammatory cytokines [19], the relationship between the properties of this fat depot and $FoxP3^{+}$ T lymphocytes, important regulators of the intensity of the inflammatory response, has long remained unexplored. In this study, we have recently revealed that greater EAT thickness in CHD patients with comparable anthropometric parameters of general

obesity (BMI, waist circumference) and severity of coronary atherosclerosis (Gensini Score index) is associated with a decrease in relative and absolute Treg content in peripheral blood, with a decrease in the absolute number of cells with intranuclear localisation of FoxP3.

The relationship between the development of inflammation and EAT thickening may be ambivalent. EAT thickening has been revealed in many inflammatory diseases including rheumatoid arthritis, psoriasis, multiple sclerosis, and human immunodeficiency virus infection [19]. Meanwhile, the conditions of inflammation provoke an increase in adipogenesis, although initially this process is protective [20].

No evidence for a relationship between FoxP3$^+$ Treg and EAT accumulation is available. However, it is known that Treg lymphocytes are able to interact with adipocytes and other immune cells in subcutaneous and visceral adipose tissue [21]. It has been evidenced that during adaptive transfer, circulating Treg cells in animals have tropism to adipose tissue, where they promote mRNA expression of genes regulating thermogenesis and the transformation of subcutaneous and epididymal white adipose tissue into beige tissue, which is beneficial in maintaining a favorable metabolic profile [21]. This is of particular importance since in healthy individuals EAT adipocytes have a phenotype close to brown adipose tissue, and with accumulation of epicardial fat they change their properties and acquire more and more features of white adipose tissue [19].

Preservation of immunoregulatory function in obesity depends on cell localisation. Specifically, the development of obesity was associated with a decrease in the relative and absolute numbers of Treg lymphocytes in visceral adipose tissue, but Treg in subcutaneous adipose tissue and spleen remained intact [12, 14]. Although we have also demonstrated an inverse relationship between the content of FoxP3$^+$ Treg lymphocytes in peripheral blood and EAT thickness, further study of the cellular composition of the stromal-vascular fraction of the EAT proper is necessary, as the number of Treg cells in it and the subcellular localisation of FoxP3 may differ from the circulatory pool. Therefore, a comparative analysis of Treg content in the circulation and epicardial fat depot with further evaluation of the relationship of these parameters with tEAT is of interest for further studies.

A number of mechanisms can be speculated that may have caused the decrease in Treg lymphocyte content in patients with thicker EAT. Patients with EAT thickness > 5 mm were characterised by increased CL-LDL and CL-LDL/CL-HDL ratio. Considering that statin therapy was more intensive in this group of patients, it is most likely associated with the baseline more unfavourable lipid profile in these patients. A recent study evidenced that increased CL-LDL was associated with pro-inflammatory changes in EAT in patients with chronic CHD [22]. It is known that elevated LDL concentrations are directly capable of causing impairment of the relative content, function, stability and migratory activity of Treg cells, thus contributing to the progression of inflammation in patients with atherosclerosis [23]. Meanwhile, the doses of statins in all patients included in our study were relatively low as a result of the development of side effects or reaching the maximum tolerated dose of the drug. Pleiotropic effects of statins are known, including in relation to Treg lymphocytes, unrelated to their hypolipidaemic action. Thus, it was evidenced that intensification of therapy with atorvastatin (increasing the dosage from 20 to 80 mg/day) led to an increase in the content of Treg lymphocytes in patients with stable CHD, while increasing the dosage of hydrophilic rosuvastatin did not cause such an effect [24]. However, it should be emphasised that culturing Treg cells with high doses of atorvastatin *in vitro* resulted in a decrease in their immunosuppressive properties and reduced expression of key molecules, including the transcription factor FoxP3 [25]. It is urgent to conduct prospective studies aimed at closer examination of hypolipidaemic and pleiotropic effects of statins against content and functional activity of Treg lymphocytes in comparison with the study of EAT in patients with CHD. In addition, patients' adherence to statin therapy was not studied in our study, but could potentially influence the obtained results.

In patients with thicker EAT, we observed a trend towards increased *PCSK9* concentrations. *PCSK9*, whose main pool is produced by hepatocytes, regulates lipid metabolism by promoting lysosomal degradation of the low-density lipoprotein receptor [26]. *PCSK9* has also been evidenced to affect the immune system: suppression of *PCSK9* gene expression resulted in increased production of IL-10, TGF-β and Treg lymphocytes [27]. Notwithstanding the important role of *PCSK9* in regulating Treg lymphocyte homeostasis and lipid metabolism, the change in its concentration in patients depending on EAT thickness was only at the trend level, and there were no correlations with the content of FoxP3$^+$-cell subpopulations at greater EAT thickness. Further studies are required to elucidate the place of *PCSK9* in the development of epicardial obesity and immunoregulatory imbalance in high cardiovascular risk patients.

With EAT thickening, we did not reveal changes in the content of key Treg lymphocyte growth factors, TGF-β and IL-10. However, Treg cell deficiency was associated with an increase in the pro-inflammatory cytokine IL-1β and the systemic inflammatory marker hsCRP, which together with the relative content of CD25$^{hi}$FoxP3$^+$ Treg lymphocytes determined the increase in EAT thickness. A study performed earlier by another group of scientists also evidenced a relationship between circulatory concentration of hsCRP and EAT thickness in patients with metabolic syndrome [28]. According to the results of the CANTOS study, inhibition of IL-1β by the monoclonal antibody drug canakinumab in patients with hsCRP concentration > 2 mg/l who had undergone myocardial infarction was accompanied by a reduction in the number of adverse cardiovascular events [29]. Considering the relationship between EAT

thickness and pro-inflammatory biomarkers revealed in this pilot study, it is important to consider the properties of the epicardial fat depot when further studying therapeutic approaches aimed at controlling inflammation in patients with atherosclerosis.

The study has several limitations, the main ones being small sample size, lack of prospective follow-up of patients, and lack of data in patients without CHD. To date, in addition, there is currently no uniform approach to assess EAT thickness in patients. Although many groups of researchers assess EAT thickness at the end of systole [30], assessment of EAT thickness at the end of diastole, such as that performed in our study, is also warranted for better correlation with the results obtained by CT and MRI [2, 17]. The borderline EAT thickness value of 5 mm used in our study is not standardised to determine the presence of epicardial obesity, but was proposed by A.G. Bertaso et al. (2013) as a result of analysis of data from the largest EAT studies by echocardiography performed in diastole [18]. The inverse correlation between EAT thickness and Treg lymphocyte content revealed in our work, however, is a reason to conduct larger prospective studies aimed at translating the findings into the clinic, since Treg lymphocytes are characterised by a high therapeutic potential [12].

## CONCLUSION

The results of the pilot study reveal a decrease of $CD4^{+}CD25^{hi}FoxP3^{+}$ Treg lymphocytes content in peripheral blood and nuclear translocation of transcription factor FoxP3 in them with increasing thickness of epicardial adipose tissue in patients with CHD. The revealed changes are not associated with anthropometric parameters of obesity and severity of coronary atherosclerosis and are associated with signs of systemic inflammation and more unfavourable serum lipid profile.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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## MICROBIOLOGY AND VIROLOGY

# BIOLOGICAL PROPERTIES AND GENETIC STRUCTURE OF CLINIC ISOLATES OF *KLEBSIELLA PNEUMONIAE* SPECIES

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## ABSTRACT

*Klebsiella pneumoniae (Kp) species complex is a genetically and ecologically diverse group of bacteria that causes a wide range of infections in humans and animals.*
***The aim of the study.*** *To carry out biological characterization and genotyping based on the study of different loci of Klebsiella pneumoniae clinical isolates.*
***Materials and methods.*** *The object of the study was three Klebsiella pneumoniae clinical isolates from different biotopes of patients from a regional children's multidisciplinary hospital. We used a complex of bacteriological, molecular genetic and bioinformatic methods. Genotyping of the isolates was carried out using the Pasteur Institute service for strains of the K. pneumoniae species complex.*
***Results.*** *All strains were susceptible to antimicrobial drugs from carbapenem (imipenem, meropenem) and tetracycline groups (tigecycline), and demonstrated high susceptibility to the Klebsiella polyvalent bacteriophage. The antibiotic resistance of the Kp ODKB-16 and ODKB-81 isolates to seven and eight antimicrobial drugs, respectively, was registered.*
*Based on the results of multilocus sequence typing, all strains were assigned to Kp1 phylogroup, K2 type and differed in sequence type, scgMLST629 profile, and KL type. Kp ODKB-16 strain was identified as ST-65, scgST-11107, KL2; ODKB-07 strain – as ST-219, scgST-6401, KL125KL114; ODKB-81 strain – as ST-86, scgST-2800, KL2KL30. The virulence gene clusters AbST, CbST, YbST, SmST, and RmST have been characterized only in the genome of the Kp ODKB-16 isolate, allowing it to be characterized as highly virulent with multidrug resistance (MDR). Additionally, genes responsible for the synthesis of types 1 and 3 fimbrial adhesins were registered in all strains, and ter operon loci were identified only in Kp ODKB-16. Resistome analysis showed that all strains had 2b genotype. Plasmids were found in the genomes of Kp ODKB-81 (IncI2) and ODKB-16 (IncFIA + IncFIB + IncHI1B).*
***Conclusion.*** *We used a comprehensive framework for genomic taxonomy of clinical isolates, which can contribute to the unification of global and regional peculiarities of the developing and microevolution of bacterial pathogens.*

***Key words:*** *Klebsiella pneumoniae species complex, genotyping, multilocus analysis, multiple antibiotic resistance, antimicrobial drugs, virulence*

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# БИОЛОГИЧЕСКИЕ СВОЙСТВА И ГЕНЕТИЧЕСКАЯ СТРУКТУРА КЛИНИЧЕСКИХ ИЗОЛЯТОВ КОМПЛЕКСА ВИДОВ *KLEBSIELLA PNEUMONIAE*

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## РЕЗЮМЕ

*Комплекс видов Klebsiella pneumoniae (Kp) представляет собой генетически и экологически разнообразную группу бактерий, вызывающую широкий спектр инфекций у людей и животных.*

***Цель исследования.*** *Биологическая характеристика и генотипирование на основе изучения разных локусов клинических изолятов Klebsiella pneumoniae.*

***Материалы и методы.*** *Объектом исследования стали три клинических изолята Kp, выделенные из разных биотопов пациентов детского многопрофильного стационара регионального уровня. В работе использован комплекс бактериологических, молекулярно-генетических и биоинформационных методов. Генотипирование изолятов проводили с использованием сервиса Института Пастера для штаммов видового комплекса K. pneumoniae.*

***Результаты.*** *Все штаммы были чувствительны к антимикробным препаратам групп карбапенемы (имипенем, меропенем) и тетрациклины (тигециклин) и демонстрировали высокую чувствительность к бактериофагу клебсиелл поливалентный. У изолятов Kp ODKB-16 и ODKB-81 отмечена антибиотикорезистентность к семи и восьми антимикробным препаратам соответственно.*

*Согласно результатам мультилокусного типирования, все штаммы отнесены к филогруппе Kp1, имели K2-тип и различались по сиквенс-типам, профилю scgMLST629 и KL-типу. Штамм Kp ODKB-16 был определён как ST-65, scgST-11107, KL2; ODKB-07 – как ST-219, scgST-6401, KL125KL114; ODKB-81 – как ST-86, scgST-2800, KL2KL30. Кластеры генов вирулентности AbST, CbST, YbST, SmST и RmST были охарактеризованы только в геноме изолята Kp ODKB-16, что позволяет охарактеризовать его как высоковирулентный с множественной лекарственной устойчивостью (МЛУ). Дополнительно у всех штаммов выявлены гены, ответственные за синтез фимбриальных адгезинов 1-го и 3-го типов, а локусы ter-оперона – только у Kp ODKB-16. Анализ резистома показал, что все штаммы имели генотип 2b. Плазмиды были определены в геномах Kp ODKB-81 (IncI2) и ODKB-16 (IncFIA + IncFIB + IncHI1B).*

***Заключение.*** *Использована комплексная схема для геномной таксономии клинических изолятов, которая может способствовать унификации глобальных и региональных особенностей возникновения и микроэволюции бактериальных патогенов.*

***Ключевые слова:*** *комплекс видов Klebsiella pneumoniae, генотипирование, мультилокусный анализ, множественная антибиотикорезистентность, антимикробные препараты, вирулентность*

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## BACKGROUND

The *Klebsiella pneumoniae* species complex is a genetically and ecologically diverse group of bacteria causing a wide range of infections in humans and animals [1]. Considering its diversity, as well as evolutionary dynamics, multidrug resistance and virulence, typing schemes for *K. pneumoniae* strains are being developed [1-3]. Controversial microbial species identification and horizontal gene transfer underlie the extensive strain heterogeneity of their phenotypes, which is of ecological, medical, and/or industrial importance [4]. Obviously, the most successful taxonomic system for genetic typing of microbial strains is the multilocus sequencing approach [5, 6]. This system is used for population biology studies and surveillance of bacterial pathogens in public health [7]. Several genotyping schemes based on different loci have been developed for the *K. pneumoniae* species complex: proper multilocus typing (MLST, multilocus sequence typing) [8], multilocus typing by main genome (cgMLST, core genome multilocus sequence typing) [2, 3], typing by *wzc* and *wzi* genes [9], as well as multilocus typing of virulence gene clusters (AbST, CbST, YbST, SmST, RmST), and antibiotic resistance (aminoglycosides, beta-lactamases, quinolones) [2, 10, 11].

It has been previously revealed that *K. pneumoniae* in 23.1 % of cases acts as an etiological factor in the development of hospital purulent-septic infections in a paediatric multidisciplinary hospital at the regional level [12]. When microbiological data from clinically relevant biotopes (blood, sputum, urine, wound contents, abdominal fluid, tracheobronchial flushes, and liquor) were examined, it was revealed that blood (32.3 %) and sputum (27.1 %) had the highest frequency of microbial isolation [12]. These clinical isolates of *K. pneumoniae* have been experimentally observed to form biofilms with varying efficacy, showing different resistance to disinfectants and antimicrobial agents (AMAs) [13-15]. It may also be further noted that traditional methods of clinical bacteriology do not allow distinguishing strains within the *K. pneumoniae* species complex, which masks the true clinical significance of each sequencing type/phylogroup and their potential epidemiological features [9]. Considering this statement, the isolation biotope and multiple AMA resistance were taken into account when selecting strains for genetic typing.

## THE AIM OF THE STUDY

Biological characterisation and genotyping based on different loci of three clinical isolates of *K. pneumoniae* isolated from different biotopes of patients of a regional paediatric multidisciplinary hospital.

## METHODS

Three isolates of *K. pneumoniae* were studied from the clinical material of patients being treated in the intensive care unit of a paediatric multidisciplinary hospital at the regional level (Irkutsk).

The isolates were determined according to morphological, tinctorial, culture, and biochemical properties using bioMérieux API systems (France) and confirmed by mass spectrometric analysis with the ultraflExtreme mass spectrometer (Bruker Daltonics, Germany) [16].

To assess susceptibility to AMA, bacterial suspension was prepared according to standard methodology with an optical density of 0.5 McFarland. AMA susceptibility was determined by disc-diffusion method using Mueller – Hinton medium (HiMedia, India); the results were analysed in accordance with the current regulations and interpretation tables of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; version 11.0, valid from January 01, 2021) [17-19]. Strains exhibiting the R criterion were categorised as resistant, I - susceptible with increased exposure, and S – susceptible. The study included discs containing aztreonam (AZT; 30 μg), amikacin (AMK; 30 μg), amoxicillin-clavulanic acid (AMC; 20-10 μg), gentamicin (GEN; 10 μg), imipenem (IPM; 10 μg), meropenem (MER; 10 μg), netilmicin (NET; 10 μg), piperacillin-tazobactam (PIT; 30 μg – 6 μg), tigecycline (TGC; 15 μg), cefepime (CEP; 30 μg), ceftazidime (CAZ; 10 and 30 μg) (NICF LLC, Russia).

To assess the susceptibility of *K. pneumoniae* isolates to phages, a commercial preparation of bacteriophage produced by SPA (Scientific Production Association) «Microgen» (Russia) with declared activity against Klebsiella – Klebsiella polyvalent bacteriophage (20 ml vials, series U387 02.2020; Ufa) was used. Determination of the level of lytic activity (LLA) of bacteriophage to *K. pneumoniae* isolates was performed by the drop method (spot-test) [20, 21].

Whole-genome sequencing was performed on NextSeq 550 (Illumina, USA) equipment using the Illumina DNA Prep Tagmentation, IDT for Illumina DNA/RNA UD Indexes Set Tagmentation, and NextSeq 500/550 High Output Kit v2.5 (300 Cycles) library preparation reagent kits, according to the manufacturer's recommendations. Pre-contig primary data were assembled using SPAdes v. 3.11.1 [22]. Contigs were aligned against the *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286 reference genome (GenBank CP003200) and corrected using MAUVE 2.4.0 (The Darling Lab, Australia) [23]. Prokka 1.14.6 (Oregon State University, USA) was used for functional annotation [24]. MOB-Typer (National Microbiology Laboratory, Canada) was used to characterize plasmids [25]. Mobile genetic elements (MGEs) (IS elements and transposons) were searched using IS-finder (France) [26].

Genotypes of isolates were determined using the Pasteur Institute database for strains of the *K. pneumoniae* species complex [27] based on MLST [8], cgMLST [2, 3], *wzc* and *wzi* gene typing [9], and MLST clusters of virulence genes (AbST, CbST, YbST, SmST, RmST) as well as antibiotic resistance (aminoglycosides, beta-lactamases, quinolones) genes [2, 10, 11].

This study was performed within the framework of the state task No. 121022500179-0 using

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## RESULTS

A brief characterization of the analyzed *K. pneumoniae* isolates is presented in Table 1. Based on AMA susceptibility results, all strains showed resistance to more than two AMAs of different groups, but were susceptible to imipenem, meropenem (carbapenem group), and tigecycline (tetracyclines). In addition, all isolates revealed high susceptibility to *Klebsiella* polyvalent bacteriophage (Table 1). It should also be mentioned that *K. pneumoniae* isolates ODKB-16 and ODKB-81 revealed resistance to seven and eight drugs, respectively, and can be defined as multidrug resistant (MDR) strains.

A summary of the genome assembly and annotation results are summarized in Table 2. The genomes of the strains were mapped to the *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286 reference genome and are shown in Figure 1. The genome sizes of the analyzed isolates varied slightly and were $5.7 \times 10^6$, $5.3 \times 10^6$, and $5.4 \times 10^6$ base pairs - b.p. for *K. pneumoniae* ODKB-16, *K. pneumoniae* ODKB-07, and *K. pneumoniae* ODKB-81, respectively. The guanine-cytosine (GC) composition was consistent with the characterization of the *K. pneumoniae* species. Plasmids belonging to different incompatibility groups were revealed in *K. pneumoniae* strains ODKB-16 and *K. pneumoniae* ODKB-81 (Table 3). It is worth noting that *K. pneumoniae* ODKB-81 carries IncI2 incompatibility group plasmid, while *K. pneumoniae* ODKB-16 carries a combined plasmid belonging to several groups simultaneously (IncFIA + IncFIB + IncHI1B), which is a characteristic feature of clinical isolates. Additionally, MOBF-type relaxase was revealed in *K. pneumoniae* ODKB-16 plasmid and conjugative mobility was predicted. Profages and integrons were not found in any isolate. MGEs were represented by insertional IS-elements and Tn-transposons. MGEs of the IS1380 and IS3 families were revealed in all three isolates in the chromosome. In addition, IS66 was revealed in the *K. pneumoniae* ODKB-7 chromosome, and IS5 and IS1 elements were revealed in *K. pneumoniae* ODKB-16. The MGEs of these families are small in length, ranging from 500 to 2000 bp. – and do not contain genes responsible for resistance or pathogenicity. Two MGEs of the IS481 and IS66 families, which do not carry resistance or virulence genes, were revealed in the *K. pneumoniae* ODKB-81 plasmid. 18 MGEs belonging to the IS1, IS3, IS4, IS5, IS6, IS21, IS66, IS110, IS481, IS630, IS1380, ISNCY, and Tn3 families were revealed in the *K. pneumoniae* ODKB-16 plasmid. One Tn3 MGE of the family included genes *pbrR* (transcription factor belonging to the MerR family), *pbrA* (P1B-type ATRase), *pbrB* (integral membrane protein), and *pbrC* (putative signaling peptidase). The *pbrTRABCD* gene cluster is thought to encode a unique, specific mechanism for lead resistance [28].

Genetic typing of *K. pneumoniae* included sequencing-type determination by MLST and cgMLST multilocus sequencing schemes, identification of markers associated with phenotypic capsule serotyping, and search for virulence and antibiotic resistance determinants.

**TABLE 1**

**BRIEF CHARACTERIZATION OF ISOLATES OF THE *K. PNEUMONIAE* SPECIES COMPLEX**

| Isolate labeling | Isolation source (date) | Antibiotics | | Genotype (23 loci*) | Polyvalent Klebsiella bacteriophage |
|---|---|---|---|---|---|
| | | Susceptibility | Resistance | | |
| *K. pneumoniae* ODKB-16 | Tracheobronchial tree (November 06, 2018) | AMC20-10, IPM10, MER10, TGC15 | AMK30, GEN10, NET10, PIT30-6, CEP30, CAZ10, CAZ30, AZT30 | 2b | 3X |
| *K. pneumoniae* ODKB-07 | Blood (June 21, 2018) | AMK30, GEN10, NET10, AMC20-10, PIT30-6, IPM10, CAZ30, MER10, TGC15 | CEP30, CAZ10, AZT30 | 2b | 3X |
| *K. pneumoniae* ODKB-81 | Sputum (October 22, 2019) | IPM10, MER10, TGC15 | AMK30, GEN10, NET10, AMC20-10, PIT30-6, CEP30, CAZ10, CAZ30, AZT30 | 2b | 4X |

**Note.** * – genotyping was performed at the following loci: blaAMPC, blaBEL, blaCARB, blaCMY, blaCTX_M, blaGES, blaIMP, blaIND, blaKPC, blaLEN, blaNDM, blaOKP_ABCD, blaOXA, blaOXY, blaPER, blaSHV, blaSME, blaTEM, blaVEB, blaVIM.

**TABLE 2**

**SUMMARY RESULTS OF GENOME ASSEMBLY AND ANNOTATION OF GENOMES OF ISOLATES OF THE *K. PNEUMONIAE* SPECIES COMPLEX**

| Characteristics | *K. pneumoniae* ODKB-16 | *K. pneumoniae* ODKB-07 | *K. pneumoniae* ODKB-81 |
|---|---|---|---|
| **Genome assembly results** | | | |
| Number of readings per sample | 18 689 678 | 23 218 431 | 8 283 075 |
| Scaffold quantity | 73 | 66 | 61 |
| N50 | 293 674 | 308 988 | 308 386 |
| **Genome annotation results** | | | |
| Genome size, b.p. | 5 693 553 | 5 333 942 | 5 368 963 |
| GC, % | 56.69 | 57.19 | 57.33 |
| Number of protein-coding sequences | 5 421 | 5 085 | 4 892 |
| rRNAs quantity | 4 | 7 | 6 |
| tRNAs quantity | 63 | 66 | 54 |
| Plasmid quantity | 1 | No | 1 |

**TABLE 3**

**BRIEF CHARACTERIZATION OF PLASMIDS OF *K. PNEUMONIAE* SPECIES COMPLEX ISOLATES**

| Characteristics | *K. pneumoniae* ODKB-16 | *K. pneumoniae* ODKB-81 |
|---|---|---|
| Size, b.p. | 359 611 | 35 021 |
| GC, % | 50.37 | 43.25 |
| Incompatibility groups | IncFIA+IncFIB+IncHI1B, rep_cluster_1254 | IncI2 |
| Probable origin | *Klebsiella pneumoniae* | *Escherichia coli* |

According to the results of MLST multilocus typing for seven genes *gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*, all strains showed a different genotype: *K. pneumoniae* ODKB-16 was identified as ST-65, *K. pneumoniae* ODKB-07 as ST-219, *K. pneumoniae* ODKB-81 as ST-86 (Table 4). All strains were assigned to the Kp1 phylogroup, *K. pneumoniae* ODKB-16 and *K. pneumoniae* ODKB-81 were K2-type according to the *wzc* and *wzi* genes, but differed in scgMLST629 profile and KL-type (Table 4).

The virulence profile of *K. pneumoniae* isolates was characterized using the virulence gene clusters AbST, CbST, YbST, SmST, and RmST typing at the Aerobactin, Colibactin, Ersiniabactin, Salmohelin, and RmST/RmpADC protein family loci, respectively. All virulence gene clusters were identified and characterized only in the genome of the *K. pneumoniae* isolate ODKB-16 (Table 5); their localization is presented on the mapped genome (Fig. 16). Thus, only the *K. pneumoniae* isolate ODKB-16 can be characterised as highly virulent with MDR.

Additionally, pathogenicity determinants that are not included in the list of marker genes and their regions that are validated for typing strains of the *K. pneumoniae* species complex were searched in the genomes [27]. It is known that strains of *K. pneumoniae* complex having serotype K2 express invasive properties [9]. Genes responsible for the synthesis of fimbrial adhesins of type 1 and type 3 (*fimA* and *mrkD*, respectively) were found in the chromosome structure of the studied *K. pneumoniae* strains. However, genes responsible for the synthesis of invasins, adhesin-pili P, α-hemolysin, thermolabile enterotoxins, and for the manifestation of the hypermucoid phenotype (*rmpA* and *magA*) were not revealed. A tellurite resistance operon ($TeO_3^{-2}$, *ter* operon) was searched within the genomes, which

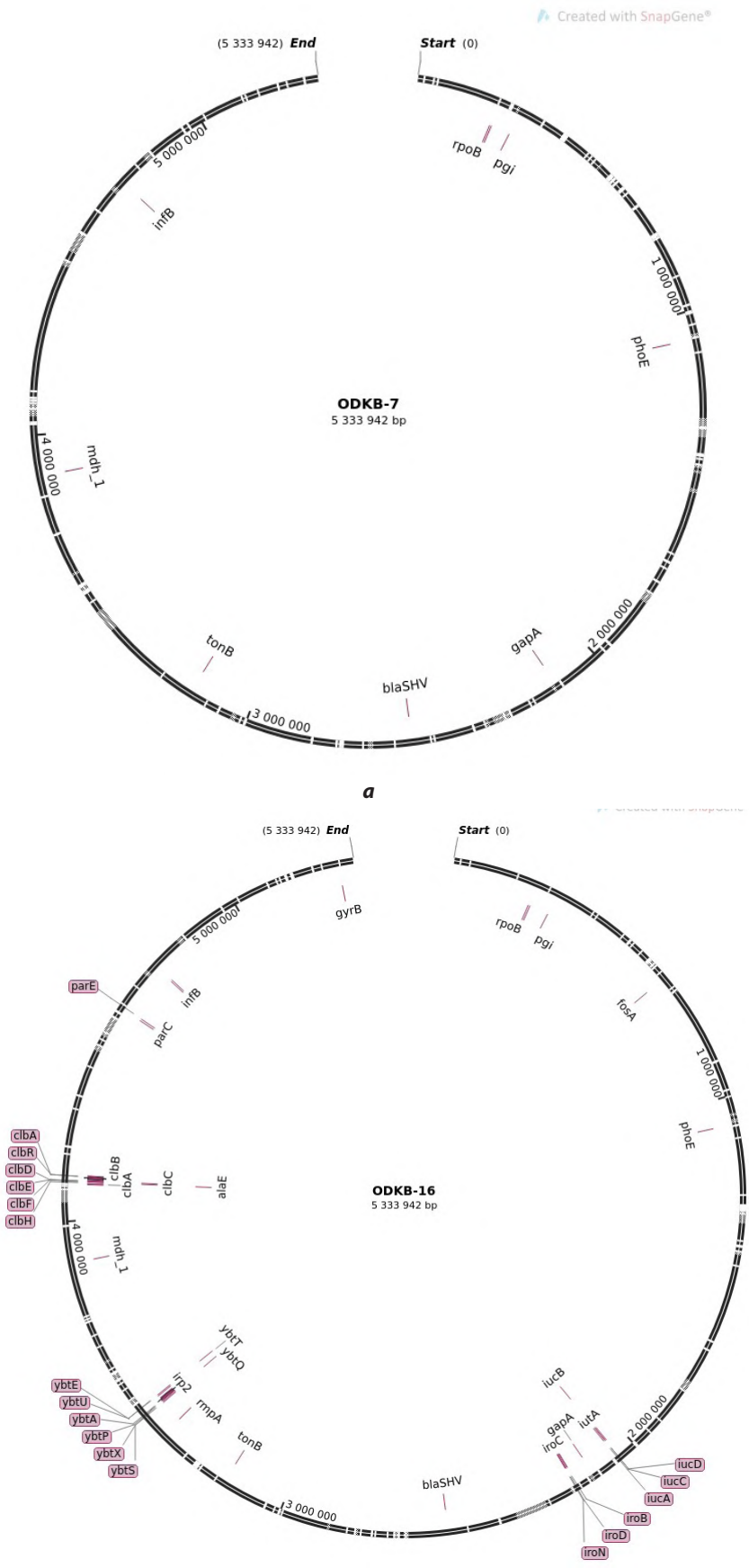


***FIG. 1.***

*Chromosome maps of K. pneumoniae ODKB-7 (а), K. pneumoniae ODKB-16 (б), K. pneumoniae ODKB-81 (в) strains mapped to the K. pneumoniae subsp. pneumoniae HS11286 reference genome. Genes identified by MLST (gapA, infB, mdh, pgi, phoE, rpoB, and tonB) as well as MLST analysis of virulence determinants (AbST, CbST, YbST, SmST, RmST) and antibiotic resistance are indicated in the footnotes*

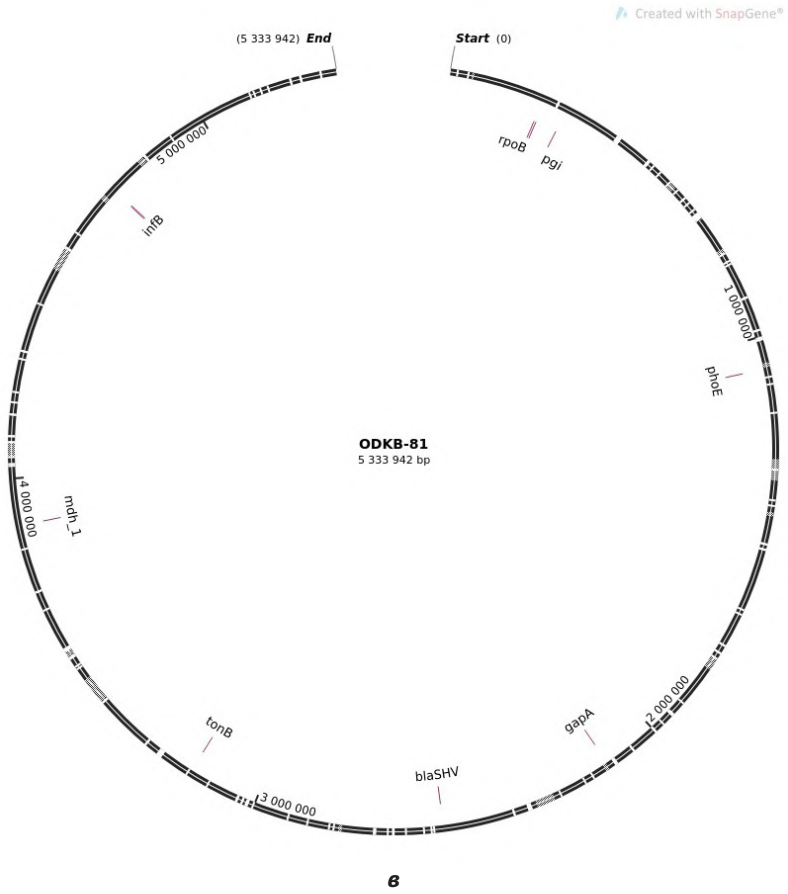


***FIG. 1.* (*continued*)**

*Chromosome maps of K. pneumoniae ODKB-7 (а), K. pneumoniae ODKB-16 (б), K. pneumoniae ODKB-81 (в) strains mapped to the K. pneumoniae subsp. pneumoniae HS11286 reference genome. Genes identified by MLST (gapA, infB, mdh, pgi, phoE, rpoB, and tonB) as well as MLST analysis of virulence determinants (AbST, CbST, YbST, SmST, RmST) and antibiotic resistance are indicated in the footnotes*

## TABLE 4

## GENETIC PROFILE OF ISOLATES OF THE *K. PNEUMONIAE* SPECIES COMPLEX

| Isolate labeling | MLST | scgMLST629 | scgMLST629_S Profile | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | LIN code | Phylogroup | Subline | Clonal group | K-type | KL-type |
| *K. pneumoniae* ODKB-16 | ST-65 | scgST-11107 | 0_0_391_0_0_0_3_1_0_0 | Kp1 | SL1471; SL322; SL65 | CG1471; CG322; CG65 | K2 | KL2 |
| *K. pneumoniae* ODKB-07 | ST-219 | scgST-6401 | 0_0_80_8_0_0_0_7_0_0 | Kp1 | SL107; SL3157 | CG219; CG3157 | no | KL125KL114 |
| *K. pneumoniae* ODKB-81 | ST-86 | scgST-2800 | 0_0_395_0_13_1_0_0_0_0 | Kp1 | SL1471; SL322; SL86 | CG1471; CG322; CG86 | K2 | KL2KL30 |

**TABLE 5**

**VIRULENCE PROFILE OF *K. PNEUMONIAE* ODKB-16 ISOLATE**

| AbST/Aerobactin/ *iucABCD, iutA** | | CbST/Colibactin/ *clbABCDEFGHILMNOPQ* | | YbST/ Yersiniabactin/ *ybtSXQPAUTE, irp2, irp1, fyuA* | SmST/Salmochelin/ *iroBCDN* | | RmST/RmpADCproteins/ *rmpACD* | |
|---|---|---|---|---|---|---|---|---|
| **Genotype** | **iuc lineage** | **Genotype** | **clb lineage** | **ICE lineage** | **Genotype** | **iro lineage** | **Genotype** | **rmp lineage** |
| ST-1 | iuc 1 | ST-13 | clb 3 | Ybt-17; ICEKp10 | ST-10 | iro 1 | ST-38 | rmp 1; KpVP-1 |

**Note.** * – gene cluster name/target product/list of genes from the gene cluster.

has been previously revealed to be closely associated with *K. pneumoniae* infection [29, 30]. All loci of this operon were only determined in the highly virulent MDR isolate of *K. pneumoniae* ODKB-16 (Fig. 16).

Resistome analysis of the studied strains using the Pasteur Institute's service for strains of the *K. pneumoniae* [27] revealed that the genome of three strains of *K. pneumoniae* contained gene clusters encoding resistance to aminoglycosides, beta-lactamases, quinolones, and had genotype 2b, determined by 23 loci encoding resistance to beta-lactam antibiotics. Additionally, *catA* and *sulA* genes encoding resistance to chloramphenicol and sulphonamides, respectively, were determined in the genomes of all strains. The *fosA* gene encoding resistance to fosfomycin was revealed only in the chromosome of *K. pneumoniae* isolates ODKB-16 and ODKB-81. Efflux pumps are considered to be one mechanism of multiple AMA resistance formation. Determinants of efflux pumps and their regulation *oqxAB, acrAB-tolC, acrZ, cusA, marAR, soxSR, rob, ramAR* (RND (Resistance-Nodulation Division) family), *mdtM, bcr* (MFS (Major Facilitator Superfamily) family), and *macAB* (ABC (ATP Binding Cassette) family) were determined in the genomes of all isolates.

## DISCUSSION

In our previous studies, opportunistic bacteria of *K. pneumoniae* species were classified as an etiological factor in the development of nosocomial generalised purulent-septic infections [12], and their ability to biofilm formation and resistance to disinfectants and AMA was observed [13-15]. Three isolates of *K. pneumoniae* with resistance to different groups of AMA were studied to determine the clinical significance and potential of the *K. pneumoniae* species complex. It should be outlined that *K. pneumoniae* strains ODKB-07 and ODKB-81 were isolated from biotopes that account for the highest frequency of isolation of opportunistic microorganisms of the *K. pneumoniae* species complex [12] – these are both blood and sputum, respectively.

The phylogenetic analysis of the *K. pneumoniae* species complex conducted by M. Hennart et al. [3] allowed to isolate seven major phylogroups Kp1-Kp7, with the most represented phylogroup Kp1 – the group of *K. pneumoniae* sensu stricto. Various studies have revealed a great diversity of sublineages (SL) and clonal groups (CG) in its phylogenetic structure, reflecting the active interest of clinical microbiologists in isolates with multidrug resistance or/and hypervirulence [1, 3, 10, 11]. Phylogenetic analyses of other *K. pneumoniae* phylogroups revealed divergent SLs, but they were not predominant; apparently clinically important sublines and clonal groups in these phylogroups have yet to be sequenced [3]. The authors also complemented the multilocus core genome analysis scheme (cgMLST, 634 loci) previously defined by S. Bialek-Davenet et al. [2]. The scgMLST629 scheme includes 629 loci and the profile combines LIN code, phylogroup (Kp), sublineage (SL), and clonal group (CG) [3].

The *K. pneumoniae* isolates ODKB-16, ODKB-07, and ODKB-81 that have been analysed in this study were assigned to genotypes ST-65, ST-219, and ST-86 based on MLST analysis. ScgMLST629 analysis revealed similarity to sublineages SL1471, SL322, and SL65 for *K. pneumoniae* ODKB-16, SL107, and SL3157 for *K. pneumoniae* ODKB-07, and SL1471, SL322, and SL86 for *K. pneumoniae* ODKB-81.

It should also be pointed out that there is a different frequency of virulence genes and AMA resistance genes being observed among the major SLs and CGs [3, 31]. Among the analyzed isolates, all loci were determined only in the *K. pneumoniae* ODKB-16 genome, according to the virulence profile proposed earlier [10, 11]. An isolate with a mean virulence score of 5 had a mean resistance score of 1 (1 = ESBL).

The emergence of highly virulent strains of *K. pneumoniae* with high resistance to antibiotics has recently forced researchers to actively study the mechanisms and factors responsible for the emergence and survival of such bacteria [29]. Considering the effect of AMA against bacteria as a cell response to an environmental stressor, the formation of resistance may be a consequence of different genetic determinants. Alternatively, cross-resistance or jointly resistance, for example, to AMA and disinfectants and/or AMA and heavy metals, may be associated with similar

genetic mechanisms for resistance phenotype formation [32, 33]. In recent experimental studies with model animals, the tellurium resistance operon, known as *ter*-operon, was revealed to be associated with pneumonia and bacteraemia caused by *K. pneumoniae* [29]. Comprehensive studies of the *ter*-operon in *K. pneumoniae* based on genomic and bioinformatic approaches revealed that the *ter*-operon was genetically independent of other plasmid-encoded virulence and antibiotic resistance loci [29]. In mouse model experiments, *ter*-operon, which is closely associated with infection, has been revealed to encode factors that resist stress induced by the local gut bugs during *K. pneumoniae* colonisation [29]. In a study modelling urinary tract infection, the role of TerC protein in resistance to ofloxacin, polymyxin B, and cetylpyridinium chloride was revealed [30], and together, the results of these studies suggest a role for *ter*-operon as a factor in persistence and stress tolerance [29, 30]. Among the *K. pneumoniae* strains analysed in this study, only in the genome of the highly virulent isolate with MDR ODKB-16 all loci of the *ter*-operon were characterized, indicating the presence of additional determinants of tolerance to environmental stressors in its genome.

## CONCLUSION

Phylogroups, sublines, and clonal groups within isolates of the *K. pneumoniae* species complex can vary considerably in their ecology and pathogenicity, and their precise definition is important in both basic research and practical public health. The genomes of three clinical isolates of *K. pneumoniae* obtained from the patients' clinical material were characterised based on different multilocus sequence typing schemes. The virulence profile was determined, MLST typing of seven genes and core genome typing with 629 genes (scgMLST62) was performed, and all isolates were assigned to the Kp1 phylogroup, K2 K-type and 2b genotype. Additionally, the *ter* operon has been characterized as a stress tolerance factor. This comprehensive species-specific scheme for genomic taxonomy of clinical isolates may be used and should help unify global and regional patterns of emergence and microevolution of bacterial pathogens.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# STUDIES OF THE EFFECT OF INDOLE AND ITS DERIVATIVE 1-BENZYLINDOLE ON THE FUNCTIONAL STATE OF THE HEART AND BLOOD VALUES OF LABORATORY RATS

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## ABSTRACT

***Background.*** *Indole and its derivatives are widely used in all areas of pharmaceutical production. The toxicometry of indole compounds has been sufficiently studied. At the same time, there is still no information on the toxic effect on individual organs and systems during long-term intake of most compounds.*

***The aim.*** *To carry out an experimental study of the toxic effect of indole and its derivative 1-benzylindole on the functional state of the heart and blood values.*

***Material and methods.*** *The work was carried out on 46 white rats, divided into groups: control group (n = 22); animals receiving indole once a day for 1 month (n = 12); animals receiving 1-benzylindole once a day for 1 month (n = 12). The substances were administered intragastrically 5 days a week. The condition of the animals was assessed by integral parameters, peripheral blood parameters and biochemical serum tests, and morphological data.*

***Results.*** *Administration of indole and 1-benzylindole caused an increase in the electrical activity of the atria, a decrease in the duration of the QRS complex, and a statistically significant decrease in blood pressure and body temperature compared to the control group. The intake of indole and 1-benzylindole decreased the number of red blood cells and hemoglobin, increased the activity of aspartate aminotransferase and alanine aminotransferase, and increased the concentrations of urea, total cholesterol and triglycerides in the blood. Against the background of long-term exposure to indole and 1-benzylindole, dystrophic disorders, hypertrophic and atrophic changes in individual fibers with a pronounced congestion of the microcirculatory vessels were revealed in the heart of rats.*

***Conclusion.*** *Indole and 1-benzylindole in case of long-term intake lead to functional disorders of the cardiovascular system, which cause the development of arterial hypertension, coronary heart disease, and atherosclerotic vascular lesions. Preventive measures in industries with possible contact with indole and its derivatives should include regular medical examinations of workers with mandatory monitoring of electrocardiography and advanced indicators of general and biochemical blood tests.*

***Key words:*** *indole, 1-benzylindole, experimental rats, functional state of the heart, blood biochemistry, heart morphology*

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# ИССЛЕДОВАНИЯ ВОЗДЕЙСТВИЯ ИНДОЛА И ЕГО ПРОИЗВОДНОГО 1-БЕНЗИЛИНДОЛА НА ФУНКЦИОНАЛЬНОЕ СОСТОЯНИЕ СЕРДЦА И ПОКАЗАТЕЛИ КРОВИ ЛАБОРАТОРНЫХ КРЫС

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## РЕЗЮМЕ

***Обоснование.*** *Индол и его производные широко используются во всех сферах фармацевтического производства. Токсикометрия индольных соединений достаточно изучена. Вместе с тем сведения о токсическом влиянии на отдельные органы и системы при длительном поступлении в организм большинства соединений до сих пор отсутствуют.*

***Цель исследования.*** *Экспериментальное изучение токсического влияния индола и его производного 1-бензилиндола на функциональное состояние сердца и показатели крови.*

***Материал и методы.*** *Работа проведена на 46 белых крысах, разделённых на группы: контрольная (n = 22); животные, получавшие индол 1 раз в день в течение 1 месяца (n = 12); животные, получавшие 1-бензилиндол 1 раз в день в течение 1 месяца (n = 12). Вещества вводили внутрижелудочно 5 дней в неделю. Состояние животных оценивали по интегральным параметрам, показателям периферической крови и биохимических анализов сыворотки, морфологическим данным.*

***Результаты.*** *Введение индола и 1-бензилиндола приводило к увеличению электрической активности предсердий, уменьшению продолжительности комплекса QRS и статистически значимо снижало артериальное давление и температуру тела по сравнению с контролем. Поступление индола и 1-бензилиндола снижало количество эритроцитов и гемоглобина, повышало активность аспартатаминотрансферазы и аланинаминотрансферазы, увеличивало концентрации мочевины, общего холестерина и триглицеридов в крови. На фоне длительного воздействия индола и 1-бензилиндола в сердце крыс выявлены нарушения дистрофического характера, гипертрофические и атрофические изменения отдельных волокон с резко выраженным полнокровием сосудов микроциркуляторного русла.*

***Заключение.*** *Индол и 1-бензилиндол в условиях длительного поступления в организм приводят к функциональным нарушениям сердечно-сосудистой системы, которые являются причиной развития артериальной гипертензии, ишемической болезни сердца, атеросклеротических поражений сосудов. Профилактические мероприятия на производствах, где возможен контакт с индолом и его производными, должны включать регулярные профосмотры работников с обязательным контролем электрогардиографии и расширенными показателями общего и биохимического анализов крови.*

***Ключевые слова:*** *индол, 1-бензилиндол, экспериментальные крысы, функциональное состояние сердца, биохимия крови, морфология сердца*

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## INTRODUCTION

The modern pharmaceutical industrial complex is characterised by the introduction of new compounds into production, often having a complex nature of action over the body. This process largely determines the change in the clinical and pathogenetic specificity of modern varieties of occupational intoxications and the emergence of new nonspecific reactions that may dominate the clinical picture of diseases. It has been revealed that the cardiovascular complex is highly sensitive to the toxic effect of chemical substances, which manifests itself in the form of dystrophic changes in the myocardium and autonomic-vascular dystonia [1, 2]. The identification of mechanisms of disorders, their nosological differentiation and diagnostics, however, is considerably complicated by the fact that lesions of the cardiovascular system with chemical etiology develop under the combined effect of a whole complex of technological factors affecting the organism. For this reason, conducting extended experimental studies makes it possible to isolate the factor of chemical exposure from the general complex of unfavourable factors and to rank its importance in the development of various pathologies of the cardiovascular system.

Indole (2,3-benzpyrrole) is an organic compound of a series of nitrogen-containing heterocycles, an ancestor of an extensive group of synthetic and naturally occurring compounds. Its content is quite high in coal tar, in some plant essential oils (e.g., in plants of the olive family, citrus). Indole and its numerous derivatives are widely used in chemical production, especially in the pharmaceutical industry. Among them, compounds with a high degree of biological activity and a wide range of pharmacological effects on the human body have been revealed [3]. Specifically, the indole derivative, 1-benzylindole, has been revealed to have cardiotropic, antiarrhythmic, anti-inflammatory and local anaesthetic activity [4].

The extensive use of indole and its compounds has been reflected in detailed studies of the toxicometric parameters of the substances [5, 6]. However, there is still no information related to the toxic effect of indole and 1-benzylindole on the cardiovascular complex at their prolonged intake into the body. Accordingly, the aim of the study was to experimentally examine the toxic effect of indole and its derivative 1-benzylindole both on the functional state of the heart and blood parameters.

## MATERIAL AND METHODS

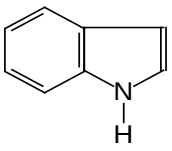
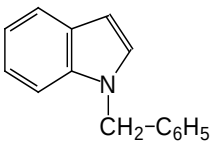
**Design and proper testing environment.** The physicochemical and toxicometric properties of indole and its derivative 1-benzylindole are summarized in Table 1.

The studies were performed on 46 white male sexually mature laboratory rats. Animals were kept and excluded from the experiment in accordance with the requirements of the guidelines of the Ministry of Health and Social Development of Russia "About the approval of the rules of laboratory practice" (No. 708-н dated August 23, 2010) and international rules «Guide for the Care and Use of Laboratory Animals» (Strasbourg, 1986).

**Ethical review.** A positive decision of the Biomedical Ethics Committee of the Research Institute of Complex Problems of Hygiene and Occupational Diseases (Minutes No. 3, § 1 dated May 12, 2022) was obtained for the experimental study. All animals underwent the necessary quarantine and were kept under standard housing conditions with natural light regime and free access to water. The rats were fed according to the standards established by the order of the Ministry of Health of the USSR № 1179 dated

**TABLE 1**

**PHYSICOCHEMICAL AND TOXICOMETRIC PROPERTIES OF THE SUBSTANCES UNDER STUDY**

| Substance | Structural formula | Gross formula | Molecular weight | Aggregate state | $DL_{50}$ (oral) | Substance hazard category |
|---|---|---|---|---|---|---|
| Indole (2,3-benzpyrrole) CAS Registry 120-72-9 |  | $C_8H_7N$ | 117.15 | Pale yellow crystalline powder with a pungent characteristic indole odour | Rats – 1200 mg/kg [7, 8] | III (moderately hazardous) |
| 1-benzylindole CAS Registry 3377-71-7 |  | $C_{15}H_{13}N$ | 207.262 | Amorphous powder of pinkish-cream color with specific indole unpleasant odor | Rats – 5800 mg/kg | IV (low-hazardous) |

October 10, 1983 "About the approval of the feed cost standards for laboratory animals in health care institutions".

**Study duration, description, and methods of enrollment.** Two series of experiments were conducted simultaneously. In the first, the rats were divided into a control group ($n$ = 11) and a group of animals receiving indole ($n$ = 12). In the second series, the rats were divided into a control group (n = 11) and a group of animals receiving 1-benzylindole ($n$ = 12). The duration of substance administration was 1 month with weekend breaks. The study substances were administered intragastrically with a metal probe at a dose of 0.1 $DL_{50}$ as a 20 % suspension on a starch gel 5 days a week, once a day. Control animals received starch gel intragastrically at appropriate doses. External examination of each animal was performed within the first hour after drug administration and every 10 days. Animals were weighed weekly and variations in feed and water intake in individual cages each day were visually observed.

Conclusions about the degree of the substances effect over the general condition of the animals were made considering the dynamics of body weight and rectal temperature measurement using TPEM-1 electrothermometer; motor activity and the value of the sum-threshold index (STI) were evaluated. Blood pressure readings were taken in animals by a noninvasive method using a LE 5001 tonometer (PANLAB, S.L. Energia, 112 08940 Cornell, Spain).

In the course of the experiment, on the $10^{th}$ and $20^{th}$ days, the rats had electrocardiogram (ECG) readings recorded in the second standard lead according to the method of L.V. Lierman (1962).

Blood sampling was performed in rats from the tail vein. The studies were performed according to a number of common clinical studies of peripheral blood: the number of erythrocytes, hemoglobin, leukocytes, analysis of leukocyte formula. The standard cyanmethemoglobin photometric method was used to determine hemoglobin content [9]. The number of erythrocytes and leukocytes was counted using the test tube method in a Goryaev chamber. Erythrocyte and leucocyte number were calculated according to standard formulae.

The following serum biochemical parameters were measured by standard methods using Vector-Best diagnostic kits (Russia): activity of serum aspartate amino transferase (AspAT), alanine aminotransferase (ALAT), glucose, urea, total cholesterol and triglycerides.

Histomorphologic examination of heart, liver, and kidney tissue was performed. A 12 % formalin solution was used as a fixative. After histological processing using the AGP-1 apparatus (Russia), the samples were embedded in paraffin. Sections 5-7 μm thick were prepared on a rotary microtome MZP-01 (Russia). The deparaffinised sections were stained with haematoxylin and eosin and Van Gieson picrofuchsin in order to reveal elastic and collagen fibres. Histological preparations were examined by light microscopy method using Nicon Eclipse E 200 (Nicon, Japan) with digital image transmission to the monitor and processing in BioVision 4.0 software.

**Statistical analysis.** Statistical processing was performed using Statistica software for Windows v. 10 (StatSoft Inc., USA) by methods of variation statistics with calculation of the following parameters: mean value, standard error of the mean, statistical significance of differences between comparison groups using the parametric Student's $t$-test ($t$) if normally distributed.

## RESULTS

The conducted studies revealed significant disturbances in the organism of experimental animals exposed to indole and 1-benzylindole. Electrocardiographic examination of the experimental group of indole-injected animals revealed a statistically significant increase in heart rate (HR) and a 10 % increase in the duration of the P wave, which is an identifier of an increase in the electrical activity of the atria (Table 2) [10]. In addition, ECG of rats that had received both indole and 1-benzylindole revealed a decrease in the duration of the QRS complex.

A statistically significant increase in the elevation of the R wave during ECG acquisition from rats that survived 1-benzylindole poisoning is also an indicator of the presence of functional cardiac abnormalities.

The indices of electrical activity of the heart muscle during indole intake correlate with a decrease in the level of general excitability of experimental animals, which was manifested in STI and motor activity changes (Table 3). The negative effect of indole and 1-benzylindole administration over physiological and behavioural reactions was expressed as an increase in the ability of the nervous system to sum up subthreshold impulses by 12 % and a decrease in the motor activity of animals in the maze: total horizontal activity by 30 %; directed horizontal and vertical activity by 40 %, integral index by 20 %. During the subacute administration a statistically significant decrease in body temperature by 0.4–0.6 °C was observed. In animals that survived indole poisoning, a statistically significant pronounced decrease in blood pressure was revealed.

Analysis of the peripheral blood components composition revealed that administration of the studied toxicants to animals resulted in relative erythropenia and decreased haemoglobin concentration (Table 4). In the quantitative characteristic of white blood, relative leucopenia was observed, developing as a result of a 21–23 % decrease in the number of paloconuclear and segmented neutrophils. The decrease in leucocyte number resulted in a relative lymphocytosis. A statistically insignificantly expressed tendency to increase by 12–15 % the number of eosinophils in the blood of indole-poisoned animals was observed.

Glycemic level is a significant diagnostic marker showing the severity of biochemical disorders in intoxication. In rats poisoned with indole, an increase in serum glucose levels was observed by 24 %, with 1-benzylindole – by 14 % (Table 4). There was a 20 % increase in blood urea concentration.

**TABLE 2**

**ELECTROCARDIOGRAM PARAMETERS OF RATS DURING SUBACUTE INTRAGASTRIC ADMINISTRATION OF SUBSTANCES (M ± M)**

| Indices | Animal groups (number) | Indole | 1-benzylindole |
|---|---|---|---|
| HR, min | Experience ($n$ = 12) | 524.0 ± 7.50* | 540.0 ± 9.54 |
| | Control ($n$ = 11) | 496.9 ± 7.74 | 528.0 ± 11.53 |
| Duration of the P wave, ms | Experience ($n$ = 12) | 21.2 ± 0.73* | 19.9 ± 0.21 |
| | Control ($n$ = 11) | 19.2 ± 0.34 | 19.7 ± 0.22 |
| P wave height, mV | Experience ($n$ = 12) | 0.12 ± 0.02 | 0.13 ± 0.02 |
| | Control ($n$ = 11) | 0.15 ± 0.019 | 0.16 ± 0.01 |
| P-Q interval duration, ms | Experience ($n$ = 12) | 44.5 ± 1.25 | 43.4 ± 0.73 |
| | Control ($n$ = 11) | 44.1 ± 1.16 | 43.1 ± 1.19 |
| QRS complex duration, ms | Experience ($n$ = 12) | 19.5 ± 0.25* | 19.4 ± 0.21** |
| | Control ($n$ = 11) | 20.6 ± 0.46 | 20.3 ± 0.21 |
| R wave height, mV | Experience ($n$ = 12) | 0.62 ± 0.07 | 0.77 ± 0.07* |
| | Control ($n$ = 11) | 0.65 ± 0.07 | 0.95 ± 0.05 |

**Note.** Differences with the control group of animals are statistically significant: * – at $p < 0,05$; ** – at $p < 0,01$.

**TABLE 3**

**INTEGRAL INDICES OF RAT CONDITION DURING SUBACUTE INTRAGASTRIC ADMINISTRATION OF SUBSTANCES (M ± M)**

| Indices | | Animal groups (number) | Indole | 1-benzylindole |
|---|---|---|---|---|
| Cumulative-threshold indicator, B | | Experience ($n$ = 12) | 4.9 ± 0.19* | 4.8 ± 0.10 |
| | | Control ($n$ = 11) | 4.4 ± 0.15 | 4.6 ± 0.10 |
| Body temperature, °C | | Experience ($n$ = 12) | 37.7 ± 0.12** | 37.5 ± 0.14*** |
| | | Control ($n$ = 11) | 38.1 ± 0.08 | 38.1 ± 0.06 |
| Blood pressure, mmHg | systolic | Experience ($n$ = 12) | 132.2 ± 5.81 | – |
| | | Control ($n$ = 11) | 136.3 ± 3.72 | – |
| | diastolic | Experience ($n$ = 12) | 81.5 ± 4.01* | – |
| | | Control ($n$ = 11) | 93.5 ± 3.65 | – |
| | mean | Experience ($n$ = 12) | 98.2 ± 3.18* | – |
| | | Control ($n$ = 11) | 108.5 ± 3.61 | – |
| Motor activity | horizontal total | Experience ($n$ = 12) | 21.8 ± 2.78* | 24.0 ± 3.91 |
| | | Control ($n$ = 11) | 28.9 ± 1.76 | 27.8 ± 2.68 |
| | horizontal directional | Experience ($n$ = 12) | 5.7 ± 1.03 | 5.3 ± 1.03 |
| | | Control ($n$ = 11) | 8.2 ± 1.04 | 7.2 ± 1.03 |
| | vertical | Experience ($n$ = 12) | 6.3 ± 1.12 | 5.3 ± 1.03* |
| | | Control ($n$ = 11) | 9.2 ± 0.77 | 8.3 ± 1.04 |
| | integral activity indicator | Experience ($n$ = 12) | 11.8 ± 1.05* | 13.0 ± 1.04 |
| | | Control ($n$ = 11) | 14.8 ± 0.32 | 13.8 ± 0.41 |

**Note.** Differences with the control group of animals are statistically significant: * – at $p < 0,05$; ** – at $p < 0,01$.

When both indole and 1-benzylindole were administered, statistically significantly increased 1.2-1.3-fold AspAT and ALAT activities were revealed in serum. The study of lipid metabolism revealed an increase in serum total cholesterol concentration by 25 % with indole administration and by 40 % with 1-benzylindole administration, and triglycerides by 45 and 50 %, respectively.

The changes revealed at the functional and biochemical levels are confirmed by the data of morphological studies of heart, liver and kidney tissue. For instance,

**TABLE 4**

**HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF BLOOD SERUM OF RATS DURING SUBACUTE INTRAGASTRIC ADMINISTRATION OF SUBSTANCES (M ± M)**

| Indices | | Animal groups (number) | Indole | 1-benzylindole |
|---|---|---|---|---|
| **Peripheral blood values** | | | | |
| HGB, g/L | | Experience ($n$ = 12) | 156.2 ± 3.87 | 148.5 ± 4.48* |
| | | Control ($n$ = 11) | 161.6 ± 3.26 | 161.0 ± 3.36 |
| RBC, $10^{12}$/L | | Experience ($n$ = 12) | 7.10 ± 0.12 | 6.65 ± 0.12* |
| | | Control ($n$ = 11) | 7.30 ± 0.11 | 7.0 ± 0.11 |
| WBC, $10^9$/L | | Experience ($n$ = 12) | 12.0 ± 1.22 | 12.5 ± 1.08 |
| | | Control ($n$ = 11) | 13.6 ± 1.47 | 15.2 ± 1.34 |
| Leukocyte formula | Banded neutrophiles, % | Experience ($n$ = 12) | 1.0 ± 0.01 | 0.9 ± 0.12 |
| | | Control ($n$ = 11) | 1.3 ± 0.12 | 1.0 ± 0.24 |
| | Segmented neutrophils, % | Experience ($n$ = 12) | 14.9 ± 1.12 | 20.1 ± 1.83 |
| | | Control ($n$ = 11) | 21.7 ± 1.84 | 24.1 ± 2.82 |
| | Eosinophils, % | Experience ($n$ = 12) | 2.8 ± 0.62 | 2.8 ± 0.62 |
| | | Control ($n$ = 11) | 2.2 ± 0.87 | 2.2 ± 0.37 |
| | Monocytes, % | Experience ($n$ = 12) | 6.1 ± 0.50 | 6.6 ± 0.37 |
| | | Control ($n$ = 11) | 7.1 ± 0.74 | 6.8 ± 0.74 |
| | Lymphocytes, % | Experience ($n$ = 12) | 76.6 ± 1.12 | 67.8 ± 1.85 |
| | | Control ($n$ = 11) | 67.6 ± 3.36 | 63.0 ± 3.48 |
| **Serum biochemical indicators** | | | | |
| AspAT activity, mmol/(h • l) | | Experience ($n$ = 12) | 1.99 ± 0.074*** | 1.73 ± 0.040*** |
| | | Control ($n$ = 11) | 1.49 ± 0.075 | 1.47 ± 0.047 |
| ALAT activity, mmol/(h • l) | | Experience ($n$ = 12) | 1.27 ± 0.115*** | 0.77 ± 0.073 |
| | | Control ($n$ = 11) | 0.62 ± 0.050 | 0.67 ± 0.044 |
| Cholesterol, mmol/L | | Experience ($n$ = 12) | 2.0 ± 0.097** | 2.0 ± 0.125*** |
| | | Control ($n$ = 11) | 1.6 ± 0.086 | 1.4 ± 0.051 |
| Triglycerides, mmol/l | | Experience ($n$ = 12) | 0.67 ± 0.054** | 0.65 ± 0.042* |
| | | Control ($n$ = 11) | 0.45 ± 0.054 | 0.43 ± 0.078 |
| Urea, mmol/L | | Experience ($n$ = 12) | 5.9 ± 0.164 | 6.7 ± 0.470** |
| | | Control ($n$ = 11) | 4.9 ± 0.122 | 5.2 ± 0.156 |
| Glucose, mmol/L | | Experience ($n$ = 12) | 7.7 ± 0.222*** | 8.0 ± 0.347 |
| | | Control ($n$ = 11) | 6.2 ± 0.261 | 7.0 ± 0.295 |

**Note.** Differences with the control group of animals are statistically significant: * – at $p < 0,05$; ** – at $p < 0,01$.

the following changes were revealed in myocardium: cardiomyocytes were thinned, with the presence of underlined striation; microcirculatory vessels were markedly full blooded; intermuscular spaces were dilated; residual traces of round cell infiltration were observed in some areas of myocardium. Due to some «homogenisation» of the middle layer of the vascular wall, thickening of vessels is observed; slight infiltration with elements of lymphoid series is revealed in them. In the vascular lumen – stasis phenomena with aggregation of aggregated erythrocytes.

The liver revealed moderately pronounced fatty dystrophy accompanied by a significant decrease in the glycogen content in the cytoplasm of hepatocytes compared to the control.

The ingestion of toxicants caused a compensatory-adaptive response of the organism, which manifested itself in diffuse proliferation of kupffer cells. Renal lesions are expressed in the form of enlarged tubules; as a result of swelling of capillary endothelium, the lumen of the Shumlansky – Bowman capsule is narrowed, the epithelium of renal tubules is swollen, loose protein masses are found in the lumen of tubules.

## DISCUSSION

Data about the toxic effect of indole and 1-benzylindole on the cardiovascular system in Russian and foreign open sources are insignificant, while indole and its derivatives are valuable industrial raw materials for the chemical and pharmaceutical industries. The conducted studies have revealed the studied xenobiotics as moderately and low hazardous at acute intragastric intake into the organism, but it does not exclude possible toxic effect, especially to heart and blood system, at subacute and chronic exposure.

The results of the conducted experiment revealed functional abnormalities in the heart. ECG monitoring of the group of animals receiving indole revealed signs of increased atrial electrical activity. The shortening of the QRS complex duration revealed on the ECG of rats poisoned by both substances compared to the control may indicate an acceleration of the depolarisation process and serve as a sign of myocardial electrical instability [11].

Lesions of the cardiovascular tract are not isolated, but develop in combination with other general and specific manifestations of the damaging effect of a chemical agent to the organism. Administration of both indole and 1-benzylindole statistically significantly decreased blood pressure and body temperature, caused changes in the indices of motor activity and STI, which demonstrates a decrease in the general excitability of the animals' organism.

Being universal, the body's defense system elicits a range of monotypic hematologic responses. The ingestion of substances in the body causes systemic damage to the processes of haemopoiesis, which manifests itself as a decrease in the number of erythrocytes and haemoglobin concentration [12, 13]. A change in the percentage of white blood elements can be considered the most typical defence reaction of the organism to toxins. In the study of white blood composition, relative leucopenia was observed due to a decrease in the number of rod-shaped and segmented neutrophils, which may be of diagnostic value. The decrease in leucocyte number resulted in a relative lymphocytosis. The number of eosinophils in the blood of animals exposed to indole poisoning is increased.

The increased level of enzyme activity in biomedical studies serves as a marker of a diseased organ, reflecting the level of its morphological destructive changes and physiological intensity of metabolism. Glycemic levels reflect the magnitude of severity of biochemical disorders in intoxication. In rats poisoned with indole and 1-benzylindole, an increase in serum glucose levels was observed, which may indicate impaired oxidation of the main energy substrates in the organs as a result of their toxic damage.

In standard clinical studies, the determination of changes in aminotransferase activity is considered one of the most highly sensitive marker assays for studying the functional status of the cardiovascular system. These enzymes are indicators of the state of organ membranes, which are characterised by their highest activity. For AspAT, the myocardium is primarily such an organ [14, 15]. Consequently, the increased AsAT activity in the serum of animals that had received indole and 1-benzylindole reflects probable membrane abnormalities accompanied by release of the enzyme into the blood. In this case, the increase in ALAT activity may signal not only hepatocyte damage, but also anabolic processes dominating in the organism of experimental animals [16].

The study of lipid metabolism revealed an increase in the concentration of total cholesterol and triglycerides in the blood serum of experimental groups of animals. The proatherogenic effect of toxicants may be one of the premorbid factors causing the development of arterial hypertension in chronic poisoning. The hypercholesterolaemia that develops under conditions of intoxication may be associated with both the ability of xenobiotics to activate a number of enzymes involved in cholesterol biosynthesis and the parallel inhibition of enzymes involved in its breakdown [17].

The 20 % increase in serum urea concentration revealed in the experiment may signal the initial stages of portal circulation disorder with the accession of renal failure.

Morphohistological studies confirmed the toxic effect towards the heart of animals poisoned with indole and 1-benzylindole. In the structure of the rat heart poisoned by these compounds, changes of dystrophic character, hypertrophic and atrophic changes of separate fibres were observed, in the microcirculatory vascular bed the walls were thickened at the expense of hypertrophy of cells of the muscular layer, full blood vessels were sharply pronounced.

**Study limitations.** The study is limited by examining the toxicological characteristics of indole and 1-benzylindole. In vivo experiments undertaken in accordance with guidelines for the protection of experimental animals limit their number as a result of animal hazards and public ethical views towards in vivo experiments.

## CONCLUSION

The results of experimental studies have revealed that indole and 1-benzylindole under conditions of long-term intake into the organism lead to heart disorders, clinical manifestations of which can be such diseases as arterial hypertension, cardiosclerosis, angina pectoris, cardiac arrhythmias, atherosclerotic lesions of blood vessels. For the purpose of prophylaxis at the production facilities where contact of employees with indole and its derivatives is possible, in addition to constant monitoring of workplaces and mandatory use of personal protective equipment, regular occupational examinations of employees with extended indices of general and biochemical blood tests and mandatory control of ECG are necessary.

**Conflict of interest**

The authors declare no apparent and potential conflicts of interest in connection with the publication of this article.

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# MOLECULAR COMPONENTS, IMMUNE AND STEM CELLS IN SOFT TISSUE REGENERATION

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## ABSTRACT

*Wound healing is a spatiotemporal and highly regulated process that is divided into four continuous and overlapping stages: hemostasis, inflammation, repair (proliferation) and remodeling. All stages are controlled by various body systems and depend on the regulatory role of immune and stem cells. Despite significant progress in understanding the cellular and molecular mechanisms of inflammation, the role of the immune microenvironment in the regeneration process remains unclear. On the one hand, the critical importance of the cellular and molecular components of the immune system in the reparative response of tissues, including the degree of scarring, restoration of structure and function of organs, has been proven, and on the other hand, little data is presented on the loss of tissue regeneration ability associated with the immune competence evolution. The review presents the key cellular and molecular mechanisms of the immune response and of the stem cells participation soft tissue repair process during their interaction with the extracellular matrix. An analysis of the latest scientific data on the participation of components of the immune microenvironment and of stem cells in soft tissue repair process was carried out based on the publications presented in Google Scholar, Medline, PubMed, Scopus and Web of Science. It has been shown that the nature of this response and its duration have a significant impact on the outcome of repair – from incomplete recovery (scarring or fibrosis) to full regeneration. It is indicated that various types of immune and stem cells take part in the soft tissue repair and remodeling processes, and their interaction must be precisely controlled. The review data may provide the basis for the development of new therapeutic approaches for soft tissue repair through immune regulation or the use of stem cells and extracellular vesicles.*

***Key words:*** *immune microenvironment, stem cells, soft tissues, regeneration, intercellular interaction*

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# МОЛЕКУЛЯРНЫЕ КОМПОНЕНТЫ, ИММУННЫЕ И СТВОЛОВЫЕ КЛЕТКИ В РЕГЕНЕРАЦИИ МЯГКИХ ТКАНЕЙ

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## РЕЗЮМЕ

*Статья посвящена 500-летию со дня рождения величайшего врача и учёного XVI века Габриэле Фаллопио (Фаллопия), революционера-морфолога, внёсшего неоценимый вклад в развитие науки, одного из основателей фундаментальной анатомии. И хотя прежде всего Фаллопий известен как анатом, описавший маточные («фаллопиевы») трубы, круг интересов учёного был гораздо шире, а вклад в анатомию – несоизмеримо более значительным. Фаллопий сделал множество важных открытий в анатомии, ряд анатомических структур носят его имя. Кроме того, Габриэле Фаллопио был талантливым педагогом и известным практикующим врачом, хирургом и фармацевтом. Особо следует отметить, что Фаллопий считал себя учеником Андреаса Везалия. Данных, подтверждающих факт личного знакомства Фаллопия и Везалия, не имеется, но есть документальное подтверждение кратковременной переписки упомянутых учёных. В своём знаменитом труде «Анатомические наблюдения» («Observationes anatomicae», 1561) Фаллопий указал на ошибки Везалия и его неточности в анатомических описаниях, подвергнув корректной критике везалиевскую «De humani corporis fabrica». Сохранился ответ Везалия с комплиментами в адрес Фаллопия как учёного. В любом случае, несомненным фактом является то, что Фаллопий был приверженцем методов Везалия в прикладной науке и преподавании анатомии и последовательно внедрял их в практику на протяжении всей своей жизни.*

***Ключевые слова:*** *история анатомии, история медицины, Габриэле Фаллопио, фаллопиевы трубы, медицинская терминология*

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The process of soft tissue regeneration after injury is accompanied by activation of various cells: platelets, neutrophils, macrophages, endothelial cells, keratinocytes and fibroblasts, as well as secretion of biologically active substances (growth factors, cytokines, chemokines and others) necessary for coordination of intercellular interactions. This process sequentially involves four coordinated steps: haemostasis, inflammation, repair (proliferation) and remodelling, which are controlled by different body systems (fig. 1) [1]. The regulatory role of immune cells in the development of the initial stages of tissue healing determines the efficiency of subsequent repair and remodelling [1, 2]. Despite significant progress in understanding the cellular and molecular mechanisms of repair, the question remains: «Why is there a tendency for incomplete healing (substitution) and scarring of damaged tissue and not complete regeneration?». The important role of the immune system in the reparative response, including the development and severity of scarring, has been proven on the one hand, and on the other hand, data on the loss of tissue regenerative capacity associated with the evolution of immune competence have been obtained. In clinical practice, the disruption and chronisation of regeneration in traumatic soft tissue defects (trauma, postoperative wounds, infected wounds) represent a serious problem. Understanding the mechanisms of regeneration, in particular the regulatory role of the extracellular matrix over tissue homeostasis, is necessary to develop ways to treat such defects. The relationship between tissue healing and immune response depends on the organ localisation of the process, the period of life of the organism (embryonic, neonatal, postnatal) and can have both negative and positive effects [3]. This review presents the major cellular and molecular mechanisms of the immune response involved in the soft tissue healing process. The nature of the immune response and its duration have a significant influence over the outcome of repair and determine the completeness of the regenerative process – incomplete (scarring or fibrosis) or complete (restitution) recovery.

The action of exogenous etiological factors of mechanical (trauma, wounding), thermal or chemical nature in soft tissues initiates primary alteration and leads to a prolonged period of increasing vascular permeability with necrosis of endothelial cells at the level of arterioles. Haemostasis with clot formation and exudative reaction with release of blood plasma and inflammatory infiltrate cells into the paravasal space are activated in the area of injury. The early transient response of increased vascular permeability is caused by the action of histamine, progesterone, leukotriene E4, serotonin, and bradykinin (fig. 1). One of the leading mediators of delayed and persistent reaction is the slow-reacting substance of anaphylaxis, which includes various leukotrienes, is secreted by mast cells and causes proteolysis of basal microvascular membranes [4]. Vascular dilation with increased endothelial permeability facilitates the migration of monocytes and neutrophils attracted by chemokines, growth factors and cytokines secreted by platelets aggregated in the lesion during haemostatic clot formation [4].

**Neutrophils**

When tissues are damaged, the cells of innate immunity provide immediate defence against potential pathogens (fig. 1), and even in the absence of pathogens, the immune response, initially triggered by molecular signals from damaged cells, can cause aseptic (sterile) inflammation [5]. Recruitment of neutrophils and the homing of these cells to the focus of injury are provided by pro-inflammatory cytokines, in particular tumour necrosis factor α (TNF-α), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), arachidonic acid derivatives – leukotrienes and prostaglandins, as well as complement components C3a and C5a [2, 6-8]. The process of effective wound healing requires the active participation of neutrophils expressing pattern-recognising receptors for microbe- and pathogen-associated molecules (MAMP/PAMP microbe-/pathogen-associated molecular pattern), as well as damage-associated molecular pattern (DAMP) [2, 6]. Such cells are phagocytised by macrophages via β2 integrins, which induces in them the release of TGF-$\beta_1$, which stimulates myofibroblast differentiation, promoting collagen synthesis and reducing the area of damage [9]. The presence of neutrophils at the injured area is generally limited to the phase of active inflammation; their longer presence in physical trauma and/or ongoing infection has a detrimental effect and prevents effective wound healing [2, 10]. Expression of the DAMP and MAMP pattern-recognising receptors by neutrophils in combination with cytokine release further enhances the inflammatory response at the injured area. In this case, the universal nuclear transcription factor «kappa-bi» (NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells), which controls the expression of immune response, apoptosis and cell cycle genes, is activated in neutrophils [11, 12]. The toxic arsenal of neutrophils directed primarily against pathogens, when released as a result of necrosis rather than apoptosis, leads to damage to the extracellular matrix, which affects blood coagulation and other mechanisms involved in wound healing [2, 8, 10, 13]. The negative influence of neutrophils can be manifested in the initiation of secondary soft tissue damage, including reperfusion, which increases the influx of these cells and the formation of persistent inflammation [14]. Another example of undesirable effects of neutrophils is the excessive formation of neutrophil extracellular traps (NET), which is considered as an inhibitor of wound healing in diabetic patients [15]. NET is an effector function of neutrophils with release of bactericidal granule components into the cytoplasm, histone modification, resulting in chromatin decondensation, nuclear envelope and cytoplasmic membrane disruption with the participation of gazdermin D protein. Subsequently, chromatin is ejected outside the cell and a structure is formed of modified nucleus chromatin surrounded by bactericidal granule proteins and cytoplasm. Uncontrolled NET formation is a provocative factor in the development of many inflammatory and autoimmune diseases [15].

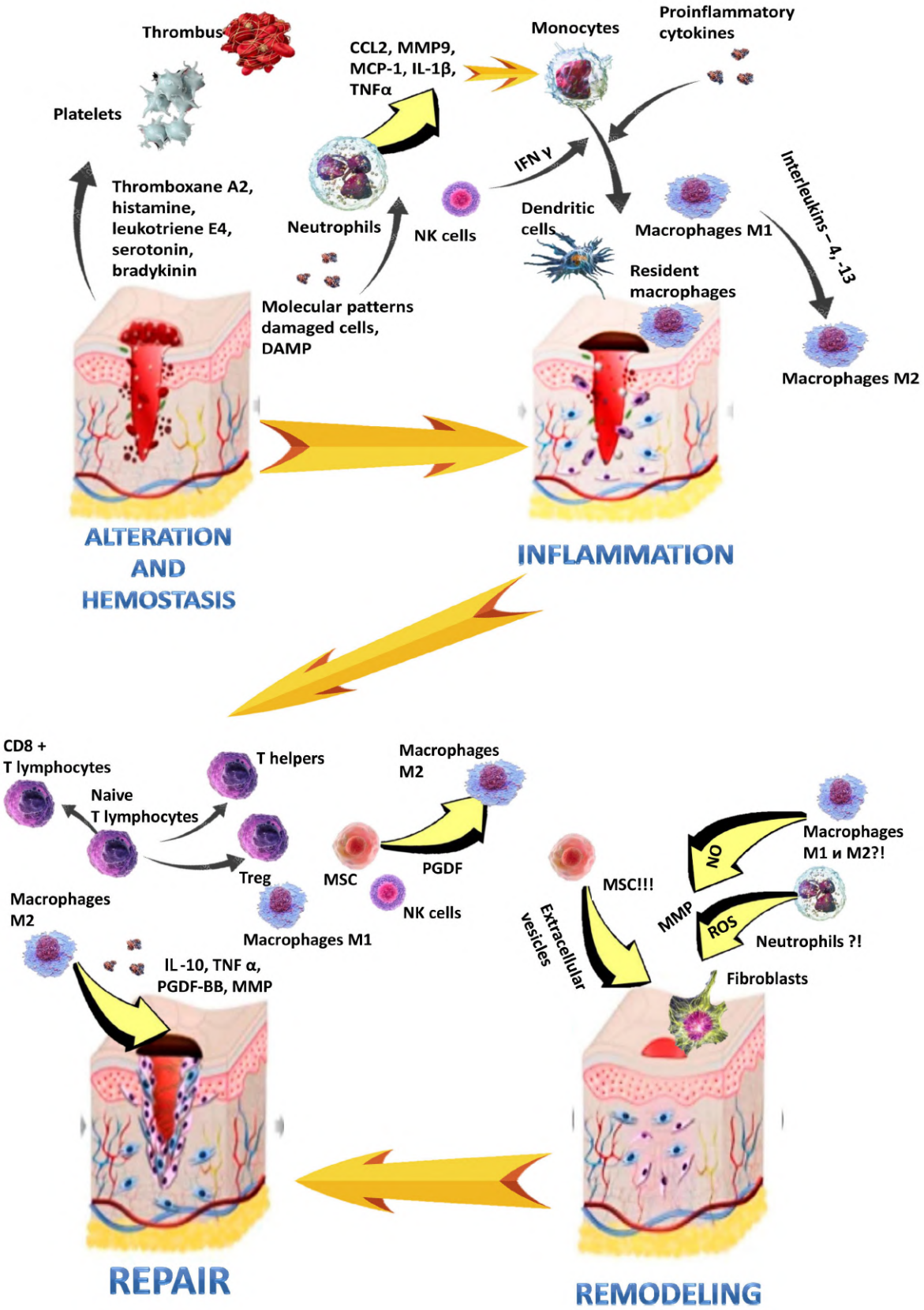


***FIG. 1.***

*Immune microenvironment and intercellular signaling during soft tissue regeneration. Stages of healing including hemostasis, inflammation, repair and remodeling. CCL2 – C-C motif ligand 2; MMP – matrix metalloproteinase; MCP-1 – monocyte chemoattractant protein-1; IL - interleukin; TNF-α - tumour necrosis factor α; IFN-γ - interferon gamma; NK – natural killer; DAMP – damage-associated molecular pattern; Treg – regulatory T-cells; MSC – mesenchymal stem cells; PDGF – platelet-derived growth factor; NO – nitrogen oxide; ROI – reactive oxygen intermediates*

The physiological role of neutrophils in wound healing is not only in the clearance of pathogens, but also in the removal of remnants of damaged cells, including red blood cells. In soft tissue repair, neutrophils have no direct effect on collagen synthesis or granulation tissue formation, but their production of cytokines, including TNF-α, can promote re-epithelialisation and wound closure [16]. Generally, the claim that neutrophils have no effect on wound healing through their regulatory effect on the synthesis of connective tissue components is rather controversial. The experimental model of aseptic wounds, for example, revealed that neutrophils take part in inflammation that ends in scarless regeneration [17]. Furthermore, a decrease in the number of neutrophils in this type of soft tissue injury without bacterial involvement correlates with high levels of anti-inflammatory cytokine interleukin (IL) 10, vascular endothelial growth factor (VEGF) and accelerated wound epithelialisation [18, 19]. VEGF secreted by neutrophils stimulates angiogenesis and promotes tissue repair [20]. There are several mechanisms to control the effect of neutrophils on the induction of repair, particularly through the scavenging of free radicals generated by hyperactivated neutrophils by superoxide dismutase-3 (SOD-3) mesenchymal stem cells (MSC) [21]. Additionally, MSCs themselves can slow neutrophil migration through TNF-6 protein gene expression and IL-10 production. Epidermal growth factor in saliva has been known to reduce neutrophil recruitment and activity, which explains the positive effect of licking wounds in animals [22]. The positive effect of neutrophils on wound healing is also their effect on cell hyperproliferation and prevention of malignisation [23]. A pronounced soft tissue inflammatory response with the presence of neutrophils that neutralise bacteria may be crucial for the control of the commensal microbiota and subsequent epitheliocyte proliferation [17]. The involvement of the powerful oxidative potential of neutrophils (production of reactive oxygen intermediates (ROIs)) is also important: in addition to their bactericidal effect, they additionally supply oxygen to proliferating cells [24]. Neutrophils support monocyte chemoattractant protein-1 (MCP-1) and chemokine ligand 3 (CCL3, C-C motif ligand 3) synthesis-mediated additional recruitment of macrophages and T lymphocytes to the injury focus [25]. During wound infection, neutrophils through the release of carboanhydrase, elimination of DAMP and MAMP alter the microenvironment, which also promotes healing processes [6]. After bacteria and necrotic tissue are removed, neutrophils undergo apoptosis or necrosis and are engulfed by macrophages via efferocytosis [26, 27]. Some neutrophils leave the injured area and return to the circulatory system by reverse migration. If neutrophils are not eliminated from the injured area, secondary necrosis develops with the release of pro-inflammatory and cytotoxic molecules. Therefore, the number and activity of these cells requires strict regulation, which is a challenge, especially in the chronic course of severe trauma.

Recently, another mechanism of neutrophil involvement in the resolution of inflammation during tissue injury has been actively discussed [2, 28]. The response in the form of migration to the inflammation location in these cells is often switched from exploratory patrolling to coordinated formation of dense clusters, so-called «swarming», which further disrupts the architecture of the surrounding tissue [28]. The response in the form of cell aggregation (their self-organisation) occurs as a result of signal transduction by paracrine chemoattractants of neutrophils themselves and primarily the inflammatory mediator leukotriene B4 [LTB4]. The mechanism of neutrophil swarm coordination is triggered in part by sustained calcium flux from necrotic tissue, which requires perception of a damage signal involving adenosine triphosphate (ATP). This «calcium alarm» signal propagates rapidly in the nascent neutrophil cluster in a contact-dependent manner via connexin-43 (Cx43, connexin 43) half-channels, which mediate the active release of ATP molecules. As a consequence, the biosynthesis of chemoattractants in the growing cluster is enhanced, which promotes coordinated cell movement and swarming. The regulatory mechanisms of swarm growth limitation are implemented with the participation of ROIs and possibly through modulation of ion channel activity [29]. There may also be similarities with neutrophil extracellular traps, the formation of which is enhanced by ATP involvement and varies with cell population density [30]. Similarly, with respect to the resolution phase, it is not clear whether monocytes or macrophages can resolve the recruitment cascade into neutrophil swarms.

## Macrophages

Infiltration of the injury focus by neutrophils and monocytes occurs in response to the appearance of chemoattractants, namely protein fragments of extracellular matrix (MCP-1) and inflammatory chemokines (CXCL8, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10). Monocytes differentiate into active macrophages, which in turn synthesise cytokines and chemokines, causing the subsequent mobilisation and recruitment of lymphocytes into the wound bed with the development of further intercellular communication (fig. 1) [1]. In the meantime, resident macrophages near capillaries in the focus of injury recognize signals of extracellular matrix and start to express purine-producing receptors on their surface, which promote the attraction of other cells [31]. Based on their different roles in the healing process, macrophages can be roughly divided into two types: inflammatory (type M1), tissue utilising and remodelling (type M2) [32, 33]. M1-type cells produce pro-inflammatory cytokines, have high phagocytic activity, can engulf apoptotically altered neutrophils and remove pathogens and dead cells, whereas M2-type cells have anti-inflammatory effects

and regulate angiogenesis, fibroblast regeneration, myofibroblast differentiation and collagen production [33]. The polarisation of macrophages into the M1 phenotype is influenced by interferon γ (IFN-γ), interleukins (IL-2, IL-3, IL-12), TNF-α, as well as by lipopolysaccharides and agonists of Toll-like receptors (TLR) [33, 34]. These cells secrete pro-inflammatory interleukins (IL-1β, IL-6, IL-12, IL-23), TNF-α, chemokines (C-X-C motif), their ligands (CXCL-9 and CXCL10) and participate in inflammatory responses. Cytokines such as IL-4, IL-10, IL-13, TGF-β and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce polarisation of M2 macrophages, which secrete anti-inflammatory molecules, promoting tissue regeneration [35].

In the early stages of injury, M1 macrophages expressing high levels of receptors for lymphocyte complex antigen 6 (Ly6c) and CC-chemokine 2 (CCR2) are the main activated phenotype in the wound microenvironment. They act predominantly as phagocytes as they remove cell fragments and pathogens and cleanse wounds of detritus. When large numbers of apoptotic neutrophils are phagocytosed, these cells can induce the transition of microenvironment components from inflammatory to proliferative [36-38]. A prospective randomised study concerning the treatment of second-degree deep burns with recombinant GM-CSF revealed that an increase in the number of tissue macrophages is associated with an enhanced local immune response and accelerated healing [39]. Although M1 macrophages have strong antibacterial activity, their persistence at the wound area may result in the secretion of matrix metalloproteinase 9 (MMP9) and tissue damage [40]. For this reason, timely polarisation of macrophages of the M1 phenotype into M2 is very important for accelerating the wound healing process; it takes place with the participation of T-helper type 2 (Th2) cytokines, apoptotic cells and with the synergistic effects of nucleotides and extracellular matrix components [41].

New data regarding the mechanism of M1 macrophage community formation in the wound bed are of interest. Swarming of these nitric oxide (NO)-producing cells may help prevent neutrophil accumulation by blocking cellular respiration and reducing the ATP:ADP (adenosine diphosphate) ratio [42]. The coordination of neutrophils resulting in their «swarming» in wounds (mentioned above) is protective, as it allows the formation of «plugs» or physically isolates sterile tissue from potential microbial invasion [43]. A community of tissue macrophages in «swarming» can actively «mask» small tissue lesions and prevent the leakage of danger signals, such as ATP in particular, to deter further tissue damage by clusters of neutrophils [44]. «Community»-type (quorum) reactions of neutrophils and macrophages reflect the group behaviour of cells and the synchronised response of the mammalian immune system to tissue damage.

Conversely, M2 macrophages at the repair stage produce cytokines that promote neutrophil apoptosis and switch from a pro-inflammatory (M1) to anti-inflammatory (M2) phenotype, while phagocytosing the debris of dead cells. The mechanism of switching from one phenotype to another has also been demonstrated to involve the scavenger receptor class B1 and/or under the control of exosomes, with the latter promoting skin wound healing by enhancing angiogenesis, re-epithelialisation and collagen formation [45, 46]. In addition to phagocytic functions, M2 phenotype macrophages participate in the regeneration process by actively synthesising TGF-β and PDGF, which influence the formation of granulation tissue [37]. M2 phenotype macrophages are responsible for the formation of new extracellular matrix with fibroblast activation and blood vessel formation. Activated fibroblasts secrete IL-1 and insulin-like growth factor (IGF-1), which participate in the initiation of the proliferative phase. Thus, macrophages play a crucial role in tissue regeneration through phenotypic polarisation and are involved in almost all its stages.

Mast cells (MS) originate from bone marrow and are located around blood vessels in the dermis, peripheral nerves, sebaceous and sweat glands [47]. They are key effectors of allergic reactions and determine the body's resistance to bacterial invasion. Upon tissue injury, activated MSs release pre- or *de novo* synthesised mediators such as histamine, serotonin, chymotrypsin, elastase and trypsin from secretory granules into the inflammatory microenvironment [47]. Histamine promotes skin wound healing by increasing the expression of basic fibroblast growth factor (bFGF) to attract macrophages and stimulate angiogenesis during inflammation [48]. The combination of trypsin with protease-activated receptors 2 (PAR2) of vascular endothelial cells causes telangiectasia and mediates neutrophil infiltration of the injured area [49]. Inflammatory mediators such as TNF-α, MMP-2 and IL-8 synthesised by MS influence neutrophil recruitment and macrophage activation [50]. The presence of excessive MS, however, can interfere with wound healing. Specifically, in type 2 diabetes mellitus, high expression of the MS receptor to IL-3 causes chronic inflammation of the skin, and prolonged activation of these cells promotes fibrosis [51]. MS also influence the proliferation stage, especially the formation of abundant granulation tissue (e.g., keloids and hypertrophic scars), angiogenesis, stimulate wound re-epithelialisation and the transition from acute to chronic inflammation [47]. It is noteworthy that different microenvironmental stimuli of the injury focus can lead to functional differences in MS, which allows us to distinguish phenotypes of actively producing anti-inflammatory mediators, secreting mediators without degranulation and degranulating mastocytes [52].

In the late stages of healing, growth factors and cytokines synthesised by MS affect the phenotype of fibroblasts, inducing the emergence of myofibroblasts that provide the transition from fibroplasia to contraction and final wound healing [53]. During tissue

remodelling, these cells can activate collagen synthesis by fibroblasts, which may be associated in part with tryptase, which has been demonstrated on human dermal fibroblasts to stimulate type I collagen synthesis [50]. In contrast, MSs produce and release potent proteolytic enzymes such as matrix metalloproteinases, initiating the degradation of the extracellular matrix. It has also been found that inhibition of histamine synthesis by MS reduces the content of hydroxyproline in granulation tissue and delays wound epithelialisation [50, 52].

**Dendritic cells (DCs)**

When soft tissue, particularly skin, is injured, various DC phenotypes are recruited to the injury focus, including epidermal Langerhans cells (ECs), dermal and plasmacytoid DCs. Dendritic cells attach to neighbouring keratinocytes using adhesion molecules, or E-cadherins; they are capable of self-renewal, and exposure to inflammatory stimuli enhances their migration and proliferation (fig 1) [54]. The formation by DC of long and complex dendritic structures between keratinocytes contributes to their rapid response to tissue damage [55]. Despite the fact that the participation of DCs in the processes of healing and regeneration of soft tissues remains a subject of study, their important role in the process of the foreign substance recognition, modulation of macrophage homeostasis and immunoregulation of regeneration processes has been revealed. In diabetic foot ulcers, healing improves with increasing amounts of DCs, indicating their positive effect over the microenvironment in the inflammatory focus [56]. Significantly accelerated healing of pressure sores in patients with high DC content in the marginal epidermis of the wound when combined with the use of drugs containing zinc [57]. DC subtypes such as $CD141^+$ stimulate CD8+ T-cell immunity by secreting IL-12 and promote differentiation of type 1 T-helper cells [54, 55, 58]. The CD1C-positive DC subtype presents antigens to $CD4^+$ cytotoxic T-cells. Furthermore, resident DCs in the injury focus express Toll-like receptors (TLR-7 and TLR-9) and induce an early inflammatory response [4]; the absence of these cells in the microenvironment negatively affects acute inflammatory responses and delays wound healing [58]. A burn wound model in DC-deficient mice showed a significant delay in the healing process associated with inhibition of early cell proliferation, low levels of TGF-β1 and neoangiogenesis in the wound beds. The essential role of DC in accelerating wound healing has thus been revealed, which is probably related to the secretion of factors that activate cell proliferation. With the development of single cell sequencing, additional opportunities are opening up to study the origin of DCs, their development, and their involvement in reparative regeneration processes in the skin.

NK cells are recruited to the focus of injury and are able to secrete immune response effectors. The key functions of these cells comprise identification of foreign, virus-infected and metabolically altered cells and induction of their apoptosis or lysis [59]. Activated cytotoxic NK cells cause lysis of target cells, including secreting death-inducing cytokines [59]. The interaction between NK cells and MSCs during regeneration has recently become an important area of studies. Undifferentiated MSCs suppress proliferation, cytokine release and cytotoxicity of NK cells, and under appropriate conditions can maintain and enhance their regenerative functions, in particular influencing neoangiogenesis and proliferation [60].

**T lymphocytes**

A subpopulation of regulatory T-cells (Treg) maintains the body's immune tolerance and inhibits the activity of potentially autoreactive T-cells, modulating the immune response and preventing autoimmune diseases. These cells also have many non-immune functions, including regulation of stem and progenitor cell activity. Tregs appear to be key cells in tissue repair and regeneration (fig. 1). It was revealed that the increased content of IL-33 synthesised by Treg influences the regulation of differentiation of fibroblast and adipocyte progenitor cells, and the decreased content of this cytokine is the main cause of unsuccessful tissue regeneration in ageing mice [61]. The presence of Treg cells in soft tissues is associated with increased regeneration rate, increased immune tolerance and induction of proliferation of resident T-cells in tissues through increased amphiregulin synthesis, resulting in an immunosuppressive microenvironment [62]. Stimulation of regeneration by Tγδ cells also through association with stem cells is not excluded [63]. Their participation in wound healing was demonstrated on the basis of the dynamics of the content of regeneration activators synthesised by them. [64]. Other types of T-cells, such as cytotoxic ($CD8^+$) and T-helper ($CD4^+$) cells, are also important activators of soft tissue regeneration. The peak content of CD4- and CD8-positive T-cells in the wound bed of the skin on days 5–10 and 7–10 after injury has been revealed and it has been demonstrated that these cells may play different regulatory roles [65]. T-helpers accompany the enhancement of regenerative processes in wounds, while cytotoxic T-lymphocytes are associated with impaired healing [66]. T-cells mediate regulation of the regeneration process through the release of a wide range of cytokines affecting both macrophages and fibroblasts.

**Stem cells (MSC)**

MSCs are also involved in the soft tissue regeneration process, affecting the wound microenvironment and regulating immune-inflammatory responses (fig. 1). At the moment, stem cell therapy is of particular interest in regenerative medicine [67]. MSC are trophoblasts that are found in virtually all tissues of the body to maintain a pool of many cell types including haematopoietic, epithelial, tumour, nerve, hepatocytes and endothelial cells. The unique features of MSC

include the ability to self-renew with asymmetric division and multidirectional differentiation and determine the modulation of tissue metabolism and regeneration, including through interaction with immune cells. While over-reactivity of innate immune cells during inflammation impairs tissue regeneration, MSCs fulfil an immunomodulatory role by producing various regulatory cytokines such as interleukins (IL-4, IL-7, IL-10), IFN-γ and prostaglandin E2 (PGE2) [68].

Signal transduction involving molecular mediators. One of the most important intercellular signals includes the regulation of stem cell activity during soft tissue regeneration using the N-terminal Janus kinase c-Jun (JNK) [69]. JNK mediates intracellular stem cell responses to stimuli from the extracellular microenvironment. JNK function is required to achieve a delicate balance between stem cell death and survival and promotes soft tissue repair and remodelling. Transplantation of preconditioned stem cells enhances soft tissue regeneration by a balanced antioxidant defence mechanism through activation of JNK signalling [70]. Signal transduction via JNK plays a critical role in the regulation of MSC differentiation into keratinocytes and promotes tissue regeneration.

The phosphatidylinositol-3-kinase, α-serine-threonine and serine-threonine protein kinase signalling pathway (PI3K/Akt/mTOR) is the mammalian target of rapamycin (mTOR) [71]. Phosphorylation of the amino acids tryptophan (Tr308) and serine (Ser473) converts Akt to an activated form that controls a multitude of cellular regulatory processes, including cell survival and cellular metabolism. The extracellular matrix provides protective mechanisms for the induction of stem cell differentiation by aberrant mTOR activation. Strategies targeting indirect mTOR activation can be used to enhance epithelial cell migration into damaged areas and accelerate soft tissue regeneration.

In soft tissue homeostasis and regeneration, Wnt/β-catenin, a signalling pathway that is involved in the regulation of tissue homeostasis by controlling cell proliferation, differentiation and apoptosis, has attracted the attention of researchers in recent decades. This extracellular signal transduction pathway has been implicated in the regulation of stem cell function and tissue repair, as well as in the progression of chronic inflammatory diseases [72]. Within the nucleus, β-catenin binds to T-cell transcription factor enhancers, thereby promoting transcription of specific genes and specific transduction of Wnt/β-catenin. Activation of the β-catenin-dependent pathway enhances the proliferation and function of epithelial and mesenchymal stem cells, promoting soft tissue regeneration [73]. Selective enhancement of Wnt/β-catenin signal transduction may be an effective strategy for the induction of soft tissue regeneration. Furthermore, from a therapeutic point of view, the role of nuclear factor erythroid 2-related factor-2 associated protein (Nrf2), which is a major mediator of redox homeostasis, has been discussed in soft tissue regeneration. This factor is expressed in a wide range of cells including stem cells, endothelial cells and fibroblasts. In damaged tissues, excess ROIs suppresses stem cell proliferation, stimulates apoptosis and impairs regeneration [74]. The activation of the antioxidant defence system in keratinocytes shows the important role of the factor Nrf2 in preventing the accumulation of ROIs; in its deficiency, epithelial wound healing is prolonged. While this evidence suggests an important role for Nrf2 signal transduction during soft tissue repair and regeneration, more studies are required to understand the function of Nrf2 in this process.

### Extracellular vesicles in the immunomodulation of tissue regeneration

Regeneration after injury requires two main conditions: the formation of a pro-inflammatory microenvironment to neutralise the injury and remove necrotised tissue and an anti-inflammatory microenvironment, with conditions for tissue regeneration through migration, proliferation and differentiation of different cell types, increased vascularisation and nutrient supply. A correlation between the increase in the number of small extracellular vesicles (sEVs), which contain various bioactive molecules, including cytokines, lipids and nucleic acids and exert paracrine effects, and the activity of cell proliferation and migration processes in the damaged tissue has been evidenced [75]. Nanoscale extracellular vesicles are involved in the regulation of intercellular communications during microenvironment formation and have attracted the attention of researchers as a promising cell-free therapeutic strategy. The MSC therapeutic activity is provided by the production of extracellular vesicles, influence on the proliferation and functional activity of cells of the microenvironment, creation of a favourable immune microenvironment in the focus of damage and enhancement of tissue regeneration [75, 76]. A study in a collagen-induced arthritis model revealed that sEVs MSC effectively inhibited IL-17A and stimulated IL-10, reducing the frequency and intensity of bone erosions, and may be a promising new cell-free therapy strategy in the treatment of rheumatoid arthritis. Small extracellular vesicles derived from human bone marrow-derived MSCs significantly reduce the expression of pro-inflammatory genes.

## CONCLUSION

Tissue damage and regeneration depend on the severity of the body's defence response involving cells of innate immunity and their active components, which is crucial for successful recovery. Spatial and temporal regulation of the functional state of immune cells involving the extracellular matrix and tissue-specific cells, including stem cells, is essential for the successful outcome of tissue regeneration.

A thorough understanding of immunomodulatory and pro-regenerative activators and their multiple functions is crucial, in particular for their successful application as therapeutic agents in developing strategies to stimulate tissue regeneration. Additional data are required for such developments, for example, if we consider macrophages of the M2 phenotype as a target, excessive activation and infiltration of the lesion focus by these cells do not contribute to tissue resistance to foreign pathogens and may impair the process of tissue healing. The mechanisms underlying this dual function are not well understood. Other immune cells, such as DC subpopulations, which have different effects on tissue regeneration depending on their functional state, or T lymphocyte populations, have similar effects. Cytotoxic $CD8^+$ T-cells have unfavourable effects towards tissue regeneration, whereas T-helpers ($CD4^+$) and Treg, to the contrary, enhance this process. The use of research technologies to study the mechanisms of coordination and functioning of various subpopulations of immune cells, in particular, sequencing of individual cells, will allow to clarify the degree of their participation in the mechanism of regulation of the microenvironment during tissue regeneration. Since the functioning of the immune system gradually decreases with age, there is a need to investigate in the elderly the relationship between the decreased ability of soft tissues to regenerate and the characteristics of their defence response. Such data may have implications for improving tissue repair with the involvement of targeted therapies.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# FEATURES OF NEUROVEGETATIVE AND HUMORAL REGULATION OF COGNITIVE ACTIVITY IN ADOLESCENTS WHEN USING ELECTRONIC DEVICES

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## ABSTRACT

***Background.*** *Studying the functional state of the body of adolescents when they preform cognitive activity using electronic devices is of great importance due to the introduction of information and computer technologies into the educational process. Identifying the characteristics of the reactivity of students' bodies when performing cognitive activities in a digital environment will contribute to both optimization of learning and health protection.*

***The aim of the study.*** *To study the autonomic regulation of heart rate, electrodermal activity, cerebral circulation and the level of cortisol in saliva when adolescents performed a cognitive test on electronic devices (tablet, laptop) and on paper-based media.*

***Materials and methods.*** *Using analysis of heart rate variability, electrodermal activity, rheoencephalography and enzyme-linked immunosorbent determination of cortisol in saliva, we examined 48 adolescents while performing a cognitive activity on electronic devices.*

***Results.*** *When adolescents perform cognitive activity using electronic devices, we can register changes in heart rate variability, electrodermal activity and cerebral circulation. Cognitive activity in a digital environment causes an increase in sympathetic effect on the heart rate with a decrease in parasympathetic activity, an increase in the integrative indicator of galvanic skin response, in vascular tone and a decrease in the cerebral blood flow intensity. One in four adolescents experiences anticipatory stimulation of the endocrine system before taking a cognitive test. Correlation analysis revealed a large number of correlations between the studied indicators both in the initial state and during cognitive activity.*

***Conclusion.*** *A large number of correlations, both in the initial state and during the cognitive test, between heart rate variability and cortisol concentration, cerebral circulation parameters indicate the preservation of a rigid system of neurovegetative and humoral regulation of heart rate when using electronic devices compared to paper-based media.*

***Key words:*** *adolescents, cognitive load, heart rate variability, electrodermal activity, cerebral circulation, cortisol*

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# ОСОБЕННОСТИ НЕЙРОВЕГЕТАТИВНОЙ И ГУМОРАЛЬНОЙ РЕГУЛЯЦИИ КОГНИТИВНОЙ ДЕЯТЕЛЬНОСТИ У ПОДРОСТКОВ ПРИ ИСПОЛЬЗОВАНИИ ЭЛЕКТРОННЫХ УСТРОЙСТВ

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## РЕЗЮМЕ

***Актуальность.*** *Изучение функционального состояния организма подростков при когнитивной деятельности с использованием электронных устройств приобретает большое значение из-за внедрения в образовательный процесс информационно-компьютерных технологий. Выявление особенностей реактивности организма обучающихся при выполнении когнитивной деятельности в цифровой среде будет способствовать как оптимизации обучения, так и здоровьесбережению.*

***Цель исследования.*** *Изучить вегетативную регуляцию сердечного ритма, электродермальную активность, мозговое кровообращение и уровень кортизола в слюне при выполнении подростками когнитивного теста на электронных устройствах (планшет, ноутбук) и бумажном носителе.*

***Материалы и методы.*** *Методами анализа вариабельности сердечного ритма, электродермальной активности, реоэнцефалографии и иммуно-ферментного определения кортизола в слюне обследовано 48 подростков при выполнении когнитивной нагрузки на электронных устройствах.*

***Результаты.*** *При выполнении подростками когнитивной нагрузки на электронных устройствах происходит изменение показателей вариабельности сердечного ритма, электродермальной активности и мозгового кровообращения. Когнитивная деятельность в цифровой среде вызывает рост симпатических влияний на сердечный ритм при снижении парасимпатической активности, увеличение интегративного показателя кожно-гальванической реакции, повышение тонуса сосудов и снижение интенсивности мозгового кровотока. Каждый четвёртый подросток испытывает упреждающее возбуждение эндокринной системы перед выполнением когнитивного теста. Корреляционный анализ выявил большое количество корреляционных связей между изучаемыми показателями как в исходном состоянии, так и в ходе когнитивной деятельности.*

***Заключение.*** *Большое количество корреляционных связей как в исходном состоянии, так и при выполнении когнитивного теста между показателями вариабельности сердечного ритма и концентрацией кортизола, параметрами мозгового кровообращения свидетельствует о сохранении жёсткой системы нейровегетативной и гуморальной регуляции сердечного ритма при использовании электронных устройств по сравнению с бумажным носителем.*

***Ключевые слова:*** *подростки, когнитивная нагрузка, вариабельность сердечного ритма, электродермальная активность, мозговое кровообращение, кортизол*

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## INTRODUCTION

At the moment (in 2019–2024), the Russian Ministry of Education is implementing a federal project «Digital Educational Environment» aimed at creating a digital educational environment, under which digital services and content for educational activities are being developed and introduced, which implies the active use of electronic devices (ED) in the education of children and adolescents. No doubt, the use of electronic devices in the educational process, on the one hand, will increase the interest, motivation and independence of students [1], but on the other hand, it can have a potentially negative impact on the functional state of the body: forced working posture, tension of the muscles of the neck, upper shoulder girdle, back [2, 3], heavy load on vision [4, 5], increased intensity of mental activity, fatigue [6]; i.e., the use of electronic learning tools for the body of schoolchildren will be physiologically challenging. There is a known fact that mental load causes changes in the activity of the nervous autonomic and endocrine systems [7, 8]. There is practically no information about neurovegetative and humoral regulation of cognitive activity in adolescents when working by means of electronic devices.

## THE AIM OF THE STUDY

To study autonomic regulation of heart rate, electrodermal activity, cerebral blood flow and cortisol levels in saliva when adolescents perform a cognitive test using electronic devices (tablet, laptop) and paper-based media.

## MATERIALS AND METHODS

The study was conducted in 2022 at one general educational organisation in Moscow; 48 students (26 boys, 22 girls) of 7$^{th}$ grades (13–14 years old; average age 13.83 ± 0.11 years) took part in it. The study was conducted in the first half of the day (from 9.00 to 14.00), at the time of the greatest activity of physiological functions. Exclusion criteria were acute infectious disease or exacerbation of chronic disease. Prior to the survey, the parents of the participants gave written informed consent for their children to participate in the study.

In order to assess heart rate variability (HRV), electrocardiogram (ECG) was recorded using a computer cardiograph «Poly-Spectr-8/E» (Neurosoft, Ivanovo). ECG was recorded in the sitting position, before cognitive task presentation (baseline) and at the 1$^{st}$–5$^{th}$, 6$^{th}$–10$^{th}$ and 11$^{th}$–15$^{th}$ minutes of test performance (load). Cardiointervalograms were studied by methods of HRV temporal and spectral analysis to assess the state of the autonomic nervous system.

To assess HRV, we used temporal indices (normal-to-normal RR intervals (RRNN), ms; standard deviation of NN intervals (SDNN), ms; root mean square of successive RR interval differences (RMSSD), ms; proportion of NN intervals differing by more than 50 ms (pNN50), %) and spectral (total power (TP), ms$^2$; high frequency (HF), low frequency (LF) and very low frequency (VLF) oscillations, ms$^2$) analyses. The LF/HF ratio was used to assess the balance of the autonomic nervous system (ANS) (the ratio of sympathetic and parasympathetic influences) [9]. Heart rate variability was assessed at 3 stages of test performance (1–5 min, 6–10 min, and 11–15 min of test performance).

**The galvanic skin response (GSR)** was assessed using the Dianel-5120 hardware and software system (Nelian CIT LLC, Moscow), recording the electrocutaneous conductance on the terminal phalanges of the 4$^{th}$ and 5$^{th}$ fingers of the right and left hands at baseline and for 15 min during the cognitive task on electronic devices and paper-based media. Galvanic skin response is an objective indicator of the level of activity of the sympathetic section of the autonomic nervous system.

**Cerebral blood flow** was studied by rheoencephalography (REG) in bifrontal (F-F) leads using the device «Rheo-Spectrum» (Neurosoft, Ivanovo). The cerebral blood flow was assessed by the following indices: a/T (%) – the ratio of the period duration of the ascending part of the wave (s) to the duration of the whole rheowave (T, s); di (%) – dicrotic index; AFR (c.u.) – amplitude-frequency ratio, which reflects blood flow per unit time.

**The response of the endocrine system** of the trainees to a cognitive task performed using various electronic devices and paper-based media was assessed by the concentration of cortisol in unstimulated saliva, which was collected in plastic disposable tubes before and after the test. Saliva samples were stored in a freezer at -20 °C until analysis. Optical density and hormone concentration values were determined using a reagent kit from DRG (USA) in StatFax 2100 enzyme immunoassay analyser (Awareness Technology, USA) and expressed in ng/mL. All analyses were performed according to the protocol of the kits, controls were within accepted limits.

During the study, students performed the Anfimov table test using different electronic devices: HP RTL8723BE laptop (HP Inc., USA) (15.6 inch screen, 1366 × 768 pixels resolution, LED backlighting with LED technology) and iPad 3 tablet (Apple Inc., USA) (9.8 inch screen, 2048 × 1536 pixels resolution at 264 ppi, LED backlighting with IPS technology).

"The Anfimov's tables" test is a task that assesses mental performance and is used to assess the stability, distribution and switching of attention in children and adolescents. The test consisted of the following: on the screen of an electronic device, the test subject was presented with a letter table, looking through it from left to right, it was necessary to find the letter highlighted in colour at the beginning of the line, marking with the help of certain keyboard keys or pressing the touch screen with a finger. The time to complete the task was 15 minutes. The control was the performance of "the Anfimov's tables" test on paper-based media, in which the subjects consistently found a letter and crossed it out with a pencil/pen. The letters to be marked during the test were given at the beginning of each line.

All adolescents completed a 15-minute "the Anfimov's tables" cognitive task on ED (tablet, laptop) and paper-based media (one trial per day). ECG, REG and GSR were recorded before (baseline) and during the 15-minute task. Saliva was collected before and after testing.

**Statistical processing** of the obtained data was performed using SPSS 26 computer programme package (IBM Corp., USA). Since the vast majority of the studied indicators did not have normal distribution, we used methods of nonparametric statistics with calculation of median (Me), lower (Q1) and upper (Q3) quartiles. Pairwise comparison of paired samples was performed using the Wilcoxon test, for comparison of independent samples the Kraskell-Wallis test and the Mann-Whitney test were used. The closeness of statistical relationship between the indicators was assessed using correlation analysis (Spearman's coefficient). Differences were considered statistically significant at $p < 0.05$.

The study of adolescents was performed in accordance with the decision of the Bioethics Committee of the Institute of Age Physiology of the Russian Academy of Education (Minutes No. 1 dated February 17, 2023).

## RESULTS

Statistical analysis revealed no differences in the studied indicators of both the response of the autonomic nervous and endocrine systems, cerebral blood flow at baseline, and the response to cognitive load (the Anfimov's proofreading test) performed by boys and girls on a laptop and tablet, so the results of the studies were combined into one group – electronic devices, and were not separated by «gender».

The results of our study showed that the intensity of mental performance (N), i.e., the total number of characters viewed when performing the test on electronic devices and paper-based media, was not statistically significantly different ($p = 0.652$) (Fig. 1). However, the total number of letters crossed out (M) and correctly selected (S) was 1.5 times greater on paper-based media than on ED ($p = 0.0001$) (Fig. 2).

When analysing the calculated indicators of mental performance, it was revealed that the value of such indicators as attention concentration (K, %), mental productivity coefficient (E, characters) and mental performance (Au, characters/s) were 1.7–2.0 times higher when working on paper-based media than on ED ($p = 0.0001$) (Fig. 3). Meanwhile, no statistically significant differences were found between ED and paper-based media in a number of indicators such as attention stability (Ku, c.u.), volume and speed of visual information processing (V and Q, characters) ($p = 0.565$-$0.652$).

Therefore, despite the fact that the intensity of mental performance when working on ED and paper-based media was almost the same, the qualitative indicators were higher on paper as a more familiar medium.

It should be noted that the most pronounced changes in time and frequency indices of heart rate variability were observed from the 11th to the 15th minute of work with Anfimov's tables regardless of the medium.

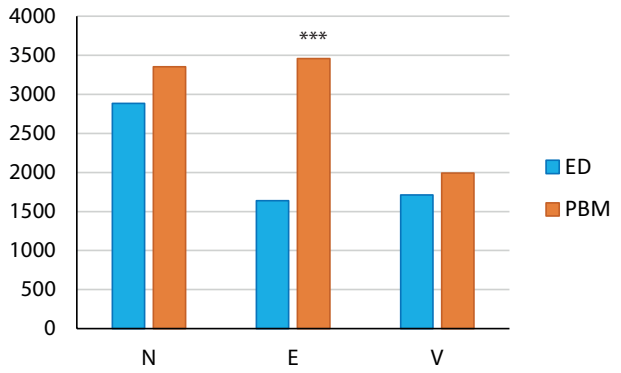


***FIG. 1.***
*Indicators of mental performance presented as median: PBM - paper-based media; N - total number of letters viewed (characters); E - mental productivity coefficient (characters); V - volume of visual information processing*

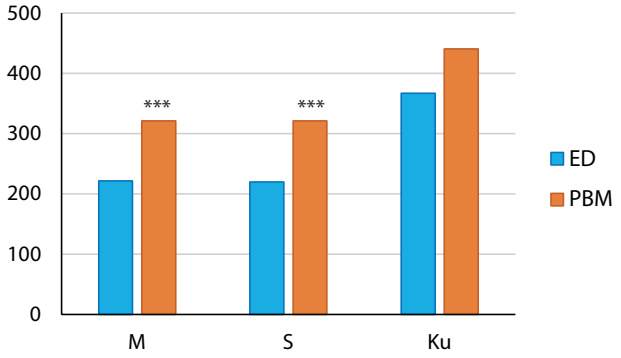


***FIG. 2.***
*Indicators of mental performance presented as median: PBM - paper-based media; M - number of crossed out letters (characters); S - number of correctly selected letters (characters); Ku - stability of attention concentration (c.u.)*

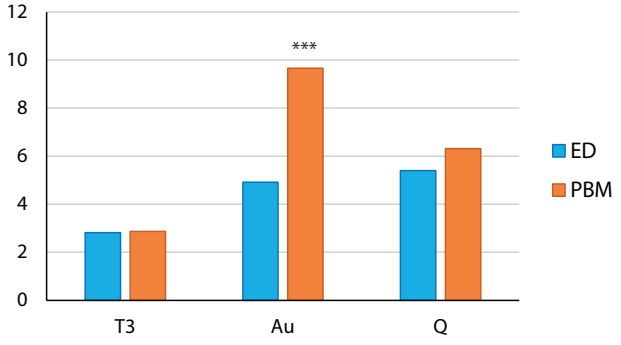


***FIG. 3.***
*Indicators of mental performance presented as median: PBM – paper-based media; T3 – accuracy (c.u.); Au – mental performance (characters/s); Q – speed of visual information processing (characters)*

Performance of the cognitive task on electronic devices in 7th grade students resulted in statistically significant decrease in total heart rate variability (decrease in TR, RRNN, SDNN values), a pronounced decrease in the activity of the parasympathetic part of the ANS (decrease in HF nu, HF% values) and a shift in the activity of the autonomic nervous system towards increased sympathetic influences (increase in LF nu, LF% and LF/HF values). HRV fluctuations in the range of VLF-spectrum reflecting neurohumoral and metabolic level of regulation did not change statistically significantly (Table 1).

During the cognitive paper-based media test in adolescents, there was an increase in low-frequency oscillations and increased sympathetic activity at the 11th–15th minute of the test (increased LF nu, LF% and LF/HF values).

GSR is used as an objective marker to assess sympathetic activation of the ANS during cognitive load. During ED work (Fig. 4a), statistically significant dynamics of changes in the amplitude (AM, μA) and integrative index of GSR activity (CA, cNp/min) of the left arm at the 11th–15th minute of cognitive load compared to baseline were revealed ($p$ = 0.023 and $p$ = 0.015, respectively). During paper-based media work (Fig. 4б), there was a statistically significant increase only in the integrative index of GSR activity (CA, sNp/min) of the left hand ($p$ = 0.017); the amplitude value (AM, μA) at baseline and after cognitive load did not change statistically significantly ($p$ = 0.087).

The results of the study of cerebral blood flow when performing the cognitive task using ED and paper-based media (Table 2) revealed that all adolescents, regardless of the type of medium, showed an increase in vascular tone in the frontal leads (A_art., Ohm) and a decrease in cerebral blood flow intensity in the frontal leads (ACF, c.u.).

The study of the functional state of the adrenal cortex in adolescents in the whole group by the level of cortisol in saliva has revealed that the performance of the cognitive task using ED and paper-based media was not accompanied by a statistically significant change in cortisol concentration ($p$ = 0.537 and $p$ = 0.311, respectively).

**TABLE 1**

**DYNAMICS OF HEART RATE VARIABILITY INDICES WHEN PERFORMING A COGNITIVE TEST ON ELECTRONIC DEVICES AND PAPER-BASED MEDIA**

| Indices | Electronic devices, Me (Q1; Q3) | | | Paper-based media, Me (Q1; Q3) | | |
|---|---|---|---|---|---|---|
| | initial state | 11-15 min. | *p* value | initial state | 11-15 min. | *p* value |
| HR, bpm | 85.40 (80.00–96.30) | 91.80 (85.15–100.65) | **0.001** | 82.10 (80.30–94.55) | 95.00 (86.95–98.55) | **0.001** |
| TP, ms² | 3174.0 (1708.5–3922.0) | 2201.0 (1492.0–3256.5) | **0.010** | 2590.0 (1862.5–3861.5) | 1832.0 (1305.0–3064.5 | 0.294 |
| VLF, ms² | 981.0 (469.5–1465.0) | 812.0 (553.5–1079.5) | 0.100 | 963.0 (586.5–1490.5) | 739.0 (470.5–1332.5) | 0.064 |
| HF, ms² | 640.0 (421.5–1116.5) | 343.0 (202.0–591.0) | **0.000** | 642.0 (166.5–1050.0) | 276.0 (166.0–425.0) | 0.133 |
| LF, ms² | 995.0 (577.0–1487.0) | 841.0 (599.0–1435.5) | 0.406 | 1005.0 (644.0–1242.0) | 791.0 (579.5–1428.0) | 0.753 |
| HF nu | 40.6 (26.90–50.70) | 29.0 (16.75–41.05) | **0.000** | 36.40 (25.40–49.90) | 29.2 (20.05–33.00) | **0.009** |
| LF nu | 59.4 (49.30–73.10) | 71.0 (58.95–83.25) | **0.000** | 63.6 (50.10–74.60) | 70.8 (67.00–79.95) | **0.009** |
| LF/HF, c. u. | 1.46 (0.97–2.72) | 2.44 (1.43–4.96) | **0.000** | 1.75 (1.02–2.94) | 2.43 (2.04–4.08) | **0.033** |
| VLF% | 33.8 (26.50–42.55) | 37.4 (31.15–43.55) | 0.532 | 42.2 (33.40–49.55) | 39.5 (31.60–44.15) | 0.421 |
| HF% | 27.5 (16.35–31.95) | 16.6 (9.85–23.55) | **0.001** | 22.7 (13.20–31.80) | 18.1 (11.30–21.20) | 0.196 |
| LF% | 37.9 (29.90–45.45) | 45.6 (34.30–51.05) | **0.030** | 36.1 (27.80–42.20) | 43.1 (36.40–50.25) | **0.039** |
| SDNN, ms | 57.0 (42.5–64.0) | 45.0 (40.0–55.5) | **0.008** | 50.0 (43.50–64.50) | 43.0 (37.00–54.50) | 0.135 |
| RMSSD, ms | 40.0 (27.0–49.5) | 30.0 (21.0–40.0) | **0.002** | 37.0 (23.50–52.00) | 25.0 (20.50–35.50) | 0.126 |
| pNN50, % | 15.7 (4.30–30.8) | 6.10 (2.20–17.75) | **0.003** | 37.0 (23.50–52.00) | 5.00 (1.85–11.50) | **0.001** |
| CV, c. u. | 8.00 (6.65–9.10) | 7.26 (5.87–8.56) | 0.056 | 7.30 (6.31–8.39) | 6.49 (5.93–8.36) | 0.507 |

**Note.** $p$ – statistically significant differences between baseline and load.

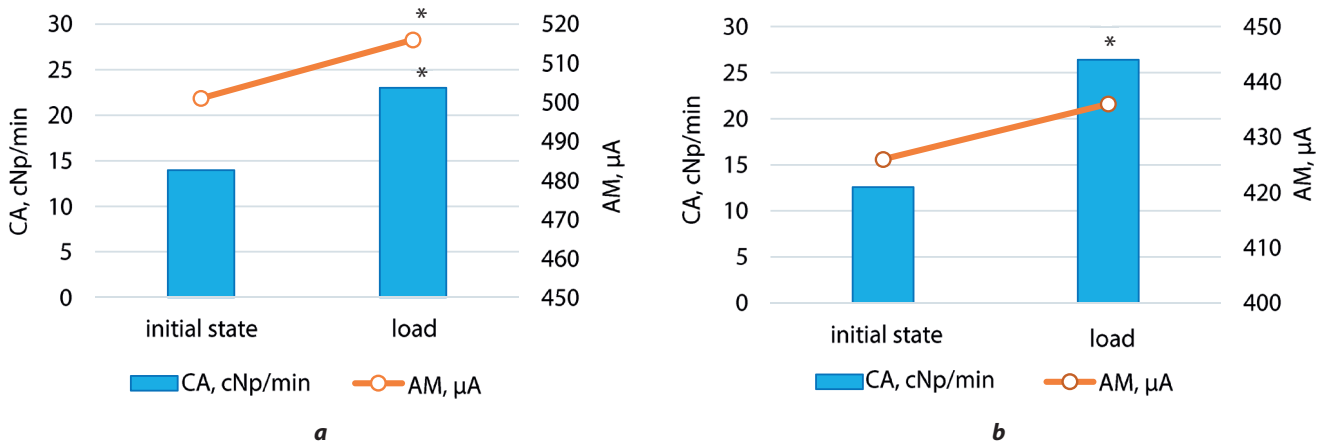


***FIG. 4.***
*Indices of cutaneous galvanic response at baseline and while performing cognitive load using electronic devices (a) and paper-based media (b): * – differences are statistically significant at $p < 0.05$ compared to the baseline state*

At the same time, individual analyses of cortisol dynamics allowed to reveal two types of response: Type I (60 % of adolescents) – increase in the level of the hormone (by 11 % when performing the ED load, 17 % – when performing on paper-based media); Type II (40 % of adolescents) – decrease in the level of cortisol (by 17 % when performing the ED load, 10 % – when performing on paper-based media) (Fig. 5).

Figures 6–8 represent the data of correlation analysis of heart rate variability, cerebral blood flow, cortisol level and mental performance during the cognitive task performed on ED and paper-based media. Before working on electronic devices, a greater number of correlations between heart rate variability indices, on the one hand, and cerebral blood flow indices and cortisol level, on the other hand, compared to the initial state before working on paper-based media (Fig. 6). The most significant correlations were observed between the total power spectrum (TP, $ms^2$) and its components (HF, LF, VLF, $ms^2$) with cerebral blood flow (AFR, c.u.) ($r$ = -0.392-0.410; $p < 0.05$) and small calibre vascular tone (di, %) ($r$ = 0.419-0.703; $p < 0.05$-0.01).


***FIG. 5.***
*Dynamics of cortisol concentration increase depending on the type of reaction to the cognitive task performed on electronic devices and paper: PBM – paper-based media; K31 – I type of reaction; K32 – II type of reaction to the cognitive task*

**TABLE 2**

**DYNAMICS OF CEREBRAL BLOOD FLOW INDICES DURING COGNITIVE TASK PERFORMANCE USING ELECTRONIC DEVICES AND PAPER-BASED MEDIA**

| Indices | Electronic devices, Me (Q1; Q3) | | | Paper-based media, Me (Q1; Q3) | | |
|---|---|---|---|---|---|---|
| | initial state | 11-15 min. | *p value* | initial state | 11-15 min. | *p value* |
| A_art, Ohm ($FF_1$) | 0.18 (0.15–0.22) | 0.13 (0.11–0.17) | **0.000** | 0.15 (0.13–0.19) | 0.10 (0.09–0.12) | **0.023** |
| AFR, c.u. ($FF_1$) | 2.63 (2.10–2.95) | 2.10 (1.82–2.43) | **0.000** | 2.38 (1.90–2.61) | 1.65 (1.39–1.92) | **0.002** |

**Note.** *p* – statistically significant difference between baseline and load.

The ED test also reveals a higher number of connections compared to the paper-based media test (Fig. 7). The most significant relationships were observed between measures of heart rate variability and large vessel tone (a/RR) ($r$ = -0.449-0.491; $p < 0.01$). Almost all correlations of cortisol levels with HRV indicators disappear, leaving only a negative association with HF spectrum waves ($r$ = -0.37; $p < 0.05$). There were no statistically significant correlations between cortisol levels and the above indices both at baseline and during the paper-based media task.

During the cognitive ED task, cortisol levels were found to correlate with such indices of mental performance as the number of correctly selected letters (S), mental productivity coefficient (E), attention concentration (R), visual information processing speed (Q), and mental performance (Au) ($r$ = 0.36-0.37; $p < 0.05$). During cognitive task on paper-based media, close correlations ($r$ = 0.63-0.66; $p < 0.05$) were revealed between the same indices of mental performance and the integrative index of GSR activity (CA of the left hand, cNp/min) reflecting sympathetic activation of the autonomic nervous system.

## DISCUSSION

The changes that we observe in the modern education of children and adolescents: the introduction of electronic devices (computers, laptops, tablets, etc.) into the educational environment – change the nature of learning activities and entail the need for physiological assessment of the optimisation of learning of the younger generation. In this regard, we conducted a study of autonomic regulation of heart rate, electrodermal activity, cerebral blood flow and cortisol levels in saliva when adolescents perform a cognitive test (Anfimov's tables) on electronic devices (tablet, laptop) and paper-based media.

According to the data of the research conducted by the Institute of Age Physiology of the Russian Academy of Education in 2019 and 2022, the duration of continuous computer use during a lesson in 7$^{th}$ grades (13.8 min) and the cumulative time of electronic media use per day during lessons (38 min) do not exceed the normative values allowed by the «Sanitary and Epidemiological Requirements for Organisations of Upbringing and Education, Recreation and Health Improvement of Children and Youth» [10]. Consequently, in this study, the 15-minute duration of adolescents' performance on the cognitive test did not exceed the acceptable values.

The results of the study revealed that mental performance (total number of letters crossed out and correctly selected, attention concentration, mental productivity coefficient, and mental efficiency) was 1.5–2.0 times higher when working on paper-based media than on ED. It is known from the literature that school-aged children and adolescents are better at solving maths problems, reading and performing tests on paper-based media than on electronic devices [11-14], i.e. most researchers agree that the traditional medium is the first choice for information perception and processing. The greater number of errors made by adolescents when working on the ED may be the result of the different viewing angle from which the subject looks at the vertical laptop screen and/or the horizontally placed task form on the desktop [15].

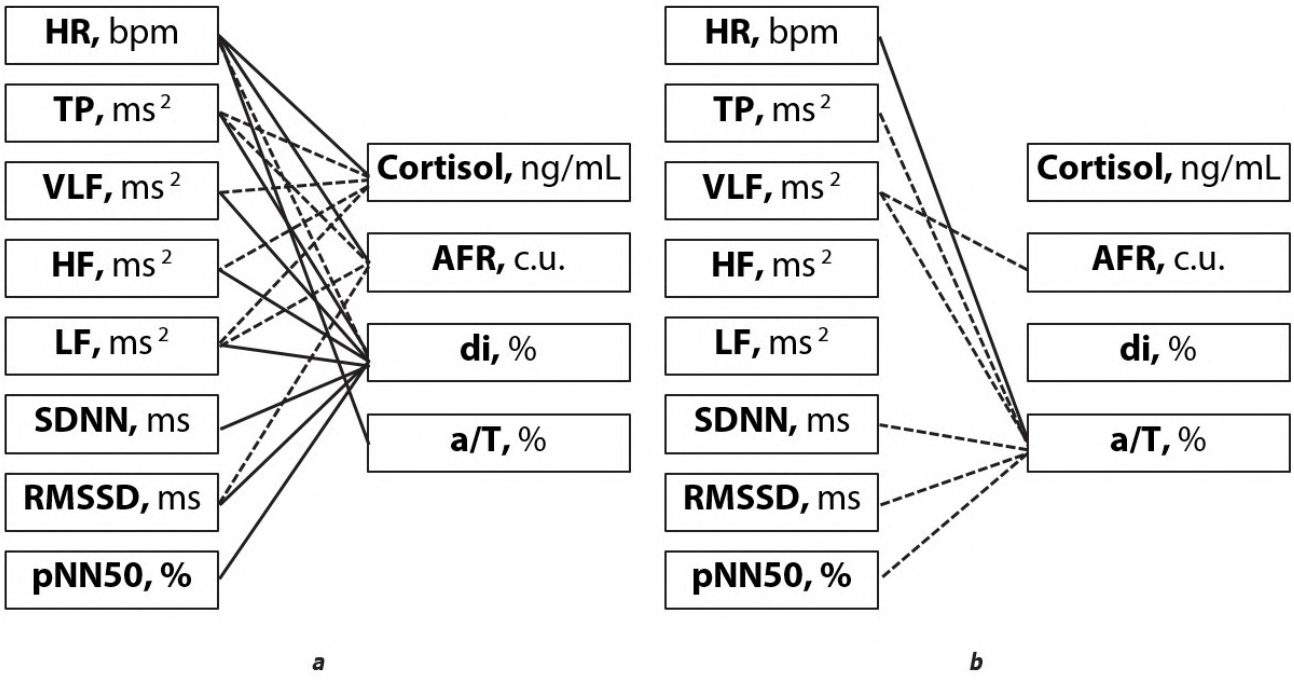


***FIG. 6.***
*Correlations of heart rate variability, cerebral blood flow and cortisol level before cognitive task performance using electronic devices (a) and paper-based media (b) ($p < 0.05$): solid line – direct relationship; dotted line – inverse relationship*

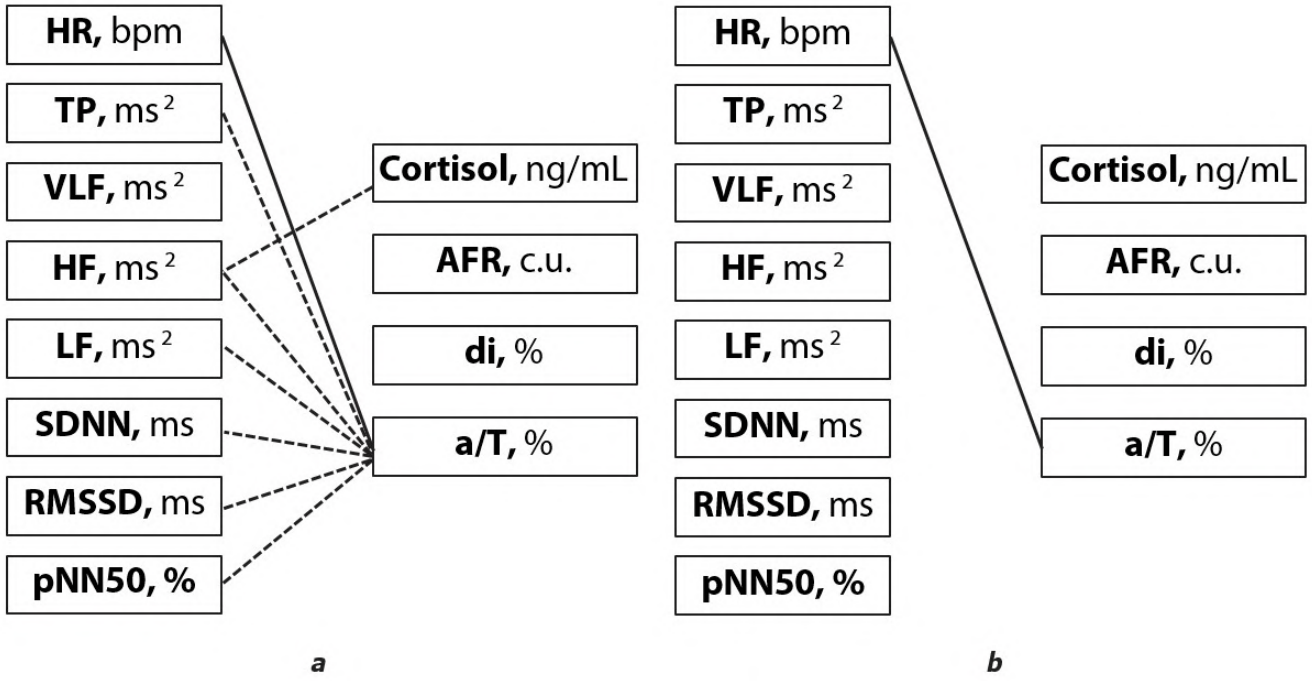


***FIG. 7.***
*Correlations of heart rate variability, cerebral blood flow and cortisol levels during cognitive task performance using electronic devices (a) and paper-based media (b) (p < 0.05): solid line – direct relationship; dotted line – inverse relationship*

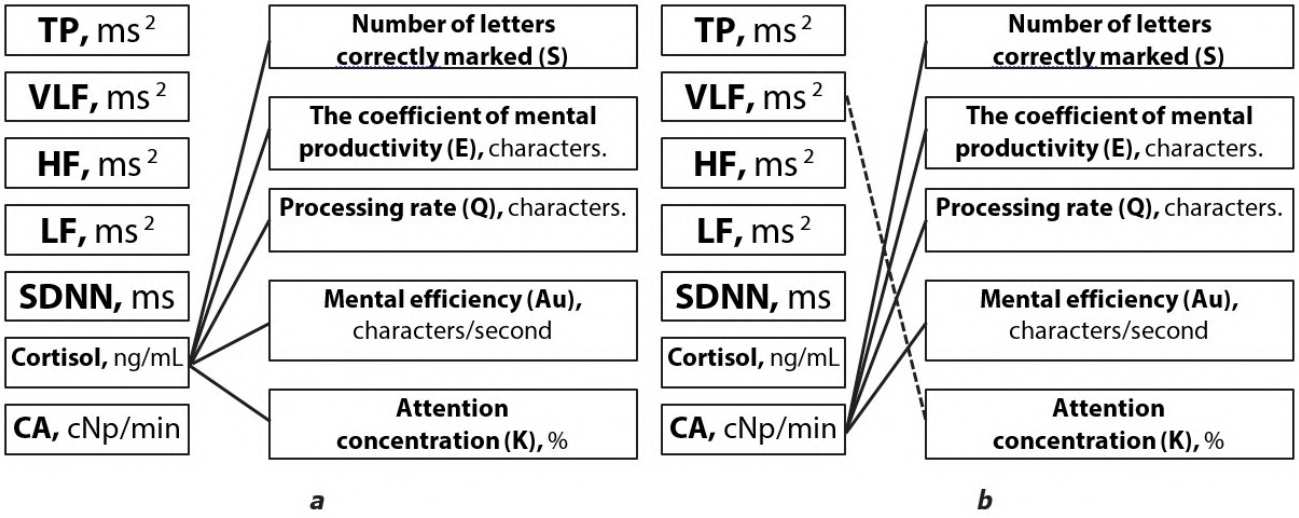


***FIG. 8.***
*Correlations of heart rate variability, cerebral blood flow and cortisol levels during cognitive task performance using electronic devices (a) and paper-based media (b) (p < 0.05): solid line – direct relationship; dotted line – inverse relationship*

Additionally, performing tasks on a computer increases the load on the systems that are involved in information processing [16], which can also affect mental performance.

Performance of a cognitive task using electronic devices in 7th grade students caused a decrease in total heart rate variability and parasympathetic activity, and an increase in sympathetic influence of the ANS. In the study by L. Mygind et al. (2018) [17] also observed a decrease in vagus nerve tone in children 10–12 years old when performing a task similar to the Anfimov's Tables test. Presumably, a rapid decrease in parasympathetic activity in response to environmental factors leads to efficient mobilisation of energy and prompt return to the resting state [18], indicating «parasympathetic flexibility» [19].

Individual analyses revealed two types of endocrine response to cognitive load: increased or decreased

cortisol levels. In this study, one in four adolescents had a higher cortisol concentration at baseline than after cognitive load. At this case, one speaks of anticipatory arousal of the endocrine system [20], which is manifested by increased cortisol levels in adult individuals before an exam [21], in children before entering school [22], which may be associated with insufficient regulatory capacity [23]. It is likely that cognitive activities in the digital environment were more stressful for some adolescents than traditional paper-based media testing, so their endocrine system was already activated before the cognitive load.

Correlation analysis revealed a large number of correlations between heart rate variability parameters with cerebral blood flow parameters, cortisol levels before working on electronic devices. According to P.K. Anokhin's theory of functional systems, coordinated interaction of individual physiological systems occurs to achieve a useful adaptive result. In other words, adaptive potential is provided by a complex of changes in several physiological systems of the organism [24].

The presence of statistically significant correlations between the total power of the spectrum and its components with cerebral blood flow (AFR) and small calibre vessel tone (di) at baseline indicates the need to maintain normal blood supply of nervous tissue, in which there is a decrease in tonic tension of cerebral arteries of small calibre, without involvement of large vessels, which is a manifestation of cerebral blood flow autoregulation [25]. The existence of correlations between the indices of the autonomic nervous system and cortisol level indicates a more generalised reaction involving not only autonomic but also humoral regulation in the situation of waiting before performing a test on electronic devices.

Changes in the number and direction of interrelations between various organismal parameters characterise the course of adaptation processes better than the absolute values of these parameters. The number of connections between the studied parameters during cognitive activity decreased, but the number of connections is greater when performing the test on ED compared to paper-based media. The most significant relationships are observed between heart rate variability indices and tone of large vessels (a/T, %). The increased oxygen demand of the brain is mainly satisfied by the increase in blood flow, which is associated with a decrease in the tone of large vessels [26]. The negative correlation between cortisol level and ANS parasympathetic activity during work with ED may indicate the mobilisation of adolescents' body reserves during adaptation to cognitive activity. This interdependence has been found in adolescents who speak publicly to unfamiliar audiences [27].

Correlations between cortisol levels and mental performance were revealed during the ED cognitive task. This is explained by the fact that cortisol in a situation of mild to moderate stress has a beneficial effect over the organism [28], increases the mobility of nervous processes, enhances brain work efficiency [29], i.e. a moderate and short-term increase in the level of cortisol contributes to the improvement of cognitive functions, in our case – an increase in mental performance. When performing a cognitive test on paper-based media, a positive relationship between the same indicators of mental performance and sympathetic activation of the ANS (according to GSR) was revealed, indicating that cognitive functions are provided by sympathetic regulation [30].

## CONCLUSION

The conducted study allowed to reveal that mental efficiency, assessed by such indicators as the total number of crossed out and correctly selected letters, concentration of attention, mental productivity coefficient, mental efficiency is 1.5–2.0 times higher when working on paper-based media than on ED, i.e. cognitive activity is more productive when performing the test on a familiar paper-based media. When working on electronic devices, there is a significant decrease in parasympathetic (as measured by HRV) and increase in sympathetic activity (as measured by HRV and GSR); when working on paper-based media, these physiological changes are less pronounced. During cognitive activity, regardless of the type of medium, there is a decrease in arterial blood flow and an increase in vascular tone in the frontal lead. The presence of a greater number of correlations both at baseline and when performing the cognitive test between HRV indices, on the one hand, and cortisol concentration and cerebral circulation indices, on the other hand, indicates the preservation of a rigid system of neurovegetative and humoral regulation of heart rhythm when using electronic devices compared to paper-based media. This may indicate a strain on the adaptation of the organism of 13-14 year old students during cognitive activities in the digital environment.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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## ONCOLOGY

# PROSPECTS FOR USING ULTRASOUND OF VARIOUS INTENSITY FOR THE TREATMENT OF PATIENTS WITH MALIGNANT BRAIN GLIOMAS

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## ABSTRACT

***Background.*** *Treatment for malignant brain gliomas includes surgery, radiation therapy, and chemotherapy with temozolomide. However, this complex treatment does not prevent tumor relapses and progression, which is caused by the activity of tumor cells and a high mutational burden. Researchers are experimenting with different intensity of focused ultrasound (FUS) in the treatment of glioblastoma (GBM). FUS has shown encouraging results in clinical studies.*

***The aim of the study.*** *This review presents brief information on the history of the development of the studied method, the results of its application in experiments and clinical trials, as well as the main possible directions for its implementation in neuro-oncology, in particular, for the treatment of glioblastomas, depending on parameters, including frequency, power, pulse duration and duty cycle.*

***Materials and methods.*** *We carried out an analysis and interpretation of existing publications; for the search, we used the PubMed database and the keywords "focused ultrasound, glioma, HIFU, LIFU", as well as Yandex and Google search engines and the same keywords in Russian.*

***Results.*** *Low-intensity FUS can be used to temporarily open the blood-brain barrier (BBB), which limits the diffusion of most macromolecules and therapeutic agents into the brain. High-intensity FUS can cause tumor ablation due to a hyperthermic effect, and also stimulate an immunological attack of tumor cells, activate sonosensitizers to exert a cytotoxic effect on tumor tissue, and can increase the sensitivity of tumors to radiation therapy. Histotripsy causes tumor ablation through acoustic cavitation.*

***Conclusion.*** *Focused ultrasound is a promising potential treatment for gliomas. Further study in the form of clinical trials should determine the optimal ultrasound parameters to achieve effective treatment for patients with malignant brain tumors.*

***Key words:*** *oncology, focused ultrasound of various intensity, pediatric oncology, neuro-oncology*

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# ПЕРСПЕКТИВЫ ПРИМЕНЕНИЯ УЛЬТРАЗВУКА РАЗЛИЧНОЙ ИНТЕНСИВНОСТИ ДЛЯ ЛЕЧЕНИЯ ПАЦИЕНТОВ СО ЗЛОКАЧЕСТВЕННЫМИ ГЛИОМАМИ ГОЛОВНОГО МОЗГА

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## РЕЗЮМЕ

***Обоснование.*** *Лечение злокачественных глиом головного мозга включает хирургическое вмешательство, лучевую терапию и химиотерапию с темозоломидом. Однако данное комплексное лечение не предотвращает рецидивы и прогрессирование опухоли, что обусловлено активностью опухолевых клеток и высокой мутационной нагрузкой. Исследователи экспериментируют с фокусированным ультразвуком (ФУЗ) различной интенсивности в лечении глиобластомы (ГБМ). ФУЗ показал обнадёживающие результаты в клинических исследованиях.*

***Цель исследования.*** *В настоящем обзоре приводятся краткие данные об истории становления указанного метода, результатах его применения в экспериментах и клинических испытаниях, а также основные возможные направления его внедрения в нейроонкологии, в частности, для лечения глиобластом, в зависимости от параметров, включая частоту, мощность, длительность импульса и рабочий цикл.*

***Методы.*** *Проведены анализ и интерпретация имеющихся публикаций, для поиска которых использовалась база данных PubMed и ключевые слова «focused ultrasound, glioma, HIFU, LIFU», а также поисковые системы Яндекс и Google и ключевые слова «фокусированный ультразвук, глиомы, HIFU, LIFU».*

***Результаты.*** *ФУЗ низкой интенсивности можно использовать для временного открытия гематоэнцефалического барьера (ГЭБ), который ограничивает диффузию большинства макромолекул и терапевтических агентов в мозг. Высокоинтенсивный ФУЗ может вызвать абляцию опухоли за счёт гипертермического эффекта, а также стимулировать иммунологическую атаку опухолевых клеток, активировать соносенсибилизаторы для оказания цитотоксического воздействия на опухолевую ткань и может повышать чувствительность опухолей к лучевой терапии. Гистотрипсия вызывает абляцию опухоли посредством акустической кавитации.*

***Заключение.*** *Фокусированный ультразвук является многообещающим потенциальным методом лечения глиом. Дальнейшее изучение в виде клинических испытаний должно определить оптимальные параметры ультразвука для достижения эффективности лечения пациентов со злокачественными опухолями головного мозга.*

***Ключевые слова:*** *онкология, фокусированный ультразвук различных интенсивностей, детская онкология, нейроонкология*

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## INTRODUCTION

Gliomas of high malignancy in adults and children represent tumours with a high degree of lethality. As a result of the diffuse nature of the growth of the disease, surgical intervention is not always possible. Chemotherapy has low efficacy due to low blood-brain barrier (BBB) permeability. As a consequence of its low radiosensitivity, radiotherapy is mainly palliative. Unsatisfactory results of treatment of malignant neoplasms of the central nervous system (CNS) for many decades have forced researchers to search for alternative options to improve treatment outcomes [1, 2], some of which are immunotherapy, oncolytic virotherapy, and vaccine therapy. Immunotherapy is an emerging therapeutic field that uses many different techniques to stimulate the body's existing immune response to a tumour. This therapy has demonstrated remarkable success in haematological malignancies unresponsive to conventional therapies such as surgery, radiation and chemotherapy. A form of immunotherapy is chimeric antigen receptor (CAR) T-cell therapy, a new form of cancer treatment approved by the U.S. Food and Drug Administration (FDA). CAR is a modified surface receptor that adds specificity to T-cells to a pre-defined target antigen exhibited on tumour cells. These synthetic receptors are retrovirally or lentivirally integrated into patient-derived T-cells and re-injected into the patient: they thus represent a special type of personalised tumour therapy. Albeit CAR-T-cell therapy is making great strides in the treatment of haematological tumours, its efficacy remains unproven in the treatment of solid tumours such as glioblastoma (GBM) [3, 4]. Studies have shown that T-cells in the tumour immune microenvironment of glioblastoma are mainly T-regulatory (Treg) cells and depleted cytotoxic T-cells [5]. In summary, CAR-T has a natural immunosuppressive effect in the treatment of glioblastoma. The combination of CAR-T and immune checkpoint inhibitors such as PD-1 antibodies may be an effective solution strategy [6]. CAR-T cell therapy, however, can cause side effects such as immune autoaggression diseases, neurotoxicity and cytokine storm. Additionally, CAR-T-cell therapy also faces many challenges such as tumour heterogeneity, off-target effect and low tumour infiltration efficiency [7].

One of the promising directions in the search for innovative preparations for the treatment of oncological diseases is the use of oncolytic (natural or genetically modified) viruses (OVs) for selective action against tumour cells and their destruction, especially as part of combination therapy. Clinical trials of oncolytic viruses in this patient group have shown promising results, with patients achieving impressive long-term clinical responses. However, OV response rates remain low. This is thought to be due to the great heterogeneity of these tumours, both in terms of molecular composition and their immunosuppressive microenvironment, leading to variability in responses [8].

Vaccine therapy against cancer has shown great promise from both preventive and therapeutic perspectives [9]. Anti-cancer vaccines are designed to target tumour-associated antigens to induce an immune response against tumours. Considering that glioblastoma-specific antigens are rare, tumour-associated antigens are the targets. Current results from clinical trials of vaccines against high grade gliomas are not very promising, the lack of GBM-specific antigen and the high heterogeneity of tumours pose challenges for GBM vaccine therapy. Therefore, the study of DMST (Diffuse midline structures tumours) at the molecular genetic level is one of the urgent tasks in neuro-oncology. Unfortunately, these treatment options are experimental due to mixed results.

Nevertheless, advances in BBB disruption using focused ultrasound have opened up new ways to deliver chemotherapeutic agents, which we believe may be a promising method that could improve treatment efficacy by increasing the concentration of systemically administered medications in the brain parenchyma, thereby potentially increasing survival in this group of patients.

## HISTORICAL BACKGROUND

The focused ultrasound (FUS) method itself is not particularly novel. It involves the machine emitting ultrasonic waves from different directions, with the waves converging on the area of interest, having the strongest effect there. Experimental study of the effect of high-intensity ultrasound on the animal organism was carried out by American researchers R.W. Wood and A.L. Loomis back in 1927 [10].

In 1942 William Fry, a veteran physicist in naval sonar research, and his brother Francis Fry began research at the Bioacoustic Research Laboratory (University of Illinois), where they developed a focused ultrasound device that focused ultrasound waves at 840 kHz (the first focused ultrasound device). They performed exposure of liver tissue to the FUS, obtaining an ablation effect. At the same time, when they attempted to irradiate the brain in animals, they failed to produce any significant damaging effect on its pathological tissue without applying the maximum possible power, which, among other things, was also accompanied by necrosis of the skin and bones of the skull. Two years later, however, they still managed to achieve selective impact of ultrasound on the brain by creating a trepanning window in the skull for its application. Already in 1950, they successfully succeeded in destroying a small focus in the human brain without damaging healthy tissue in a patient with Parkinson's disease [10].

In the 1950s the role of ultrasound as a possible method of treating tumours in humans and animals was actively studied by a research team led by Andrei K. Burov with the participation of a group of oncologists headed by Academician Nikolai N. Blokhin. In fact,

these researchers were pioneers in the use of high-intensity ultrasound in oncology. The results of their work are still known to specialists all over the world, they formed the basis for a number of subsequent studies. The ultrasonic wave sources they developed were characterised by high power: they used unfocused ultrasonic beams with a frequency of 1.5 MHz 200–500 W. Brown-Pearce carcinomas that had been transplanted into the testicles of rabbits were treated with such waves once. In 60–80 % of cases, the tumour either disappeared completely or underwent scar degeneration. Most notably, regression of not only the primary tumour but also its metastases, which were not affected by ultrasound, was observed, which could probably be explained by an immune mediated effect. In this case, repeated tumour grafting in experimental animals became impossible. The team headed by A.K. Burov also tested this method in the clinic: 10 people with terminal stage melanoma were treated, some of them showed complete disappearance of the tumour [10]. To date, Russian physicists are actively continuing research in this area. An example is the laboratory of industrial and medical ultrasound at the Department of Acoustics, Faculty of Physics, N.V. Lomonosov Moscow State University (headed by Oleg A. Sapozhnikov, Vera A. Khokhlova). One of the main directions of activity of this laboratory is the study of powerful focused ultrasound in therapy and non-invasive surgery, including modelling and measurement of nonlinear fields of medical ultrasound sources, investigation of the mechanisms of ultrasound effect on biological tissues, development of powerful multi-element phased arrays for ultrasound surgery [8].

It should also be emphasised that currently researchers from the Laboratory of Medical and Industrial Ultrasound at the Department of Acoustics, Faculty of Physics, N.V. Lomonosov Moscow State University, together with colleagues from the University of Washington (Seattle) as part of an international team are conducting studies aimed at the impact of focused ultrasound radiation on various tissues and organs inside the human body, non-invasively, i.e. without conventional surgical intervention. Meanwhile, this actively developing scientific trend has existed for about a quarter of a century and rather quickly moved from purely laboratory experiments to clinical use. For instance, in the last ten years it has become especially relevant as a result of the use of high-intensity FUS, in which researchers have learned to cause thermal necrosis of tumour tissues in the prostate, kidney, liver, breast and even in the brain, and the list is not exhausted [11].

In this regard, we would like to note the fact that the undoubted priority of domestic researchers in this area of medical science was signalled by the following international event. For her contribution to interdisciplinary research in biomedical and physical acoustics, V.A. Khokhlova was honoured in 2023 with the Helmholtz-Rayleigh Silver Medal of the Acoustical Society of America. With this award, the authoritative acoustics community recognised many years of successful studies by Vera A. Khokhlova and her associates concerning the use of nonlinear acoustic effects in medical applications of high-intensity ultrasound.

At the end of the XX century, namely in 1991, A. Gutkelch, K. Hininen, et al. reported about the treatment of malignant brain tumours with focused ultrasound hyperthermia and irradiation, and already in 1992 K. Hininen, et al. first proposed to use non-invasive focused ultrasound combined with magnetic resonance imaging (MRI) to control and monitor tissue damage. In doing so, the term MRI-guided focused ultrasound (MRgFUS) was first proposed. In 2001, the first International Society for Therapeutic Ultrasound (ISTU) was established to expand and disseminate knowledge of therapeutic ultrasound to the scientific and medical community. During the same year, G. Clement and K. Hininen demonstrated noninvasive focusing through the human skull using a phased array and a CT-based planning algorithm, and in 2006, M. Kinoshita, et al. demonstrated antibody delivery across the blood-brain barrier in brain tumours using MRgFUS [10].

Considerable experience in the use of ultrasound ablation for the treatment of neoplasms of different localisation: prostate cancer, breast cancer, liver cancer, bone tissue tumours, brain tumours has been accumulated in China [10, 12-14].

## EXPOSURE TO HIGH-INTENSITY ULTRASOUND

Currently, focused guided ultrasound under the control of MRI or ultrasound is used to ablate certain areas of the brain in the treatment of a number of neurological diseases: essential tremor, Parkinson's disease with predominant tremor, neuropathic pain - as well as for stopping bleeding, crushing kidney concretions. Additionally, thermal ablation with high-intensity ultrasound is officially approved in various countries for the treatment of malignant and benign tumours of the breast, prostate, liver, as well as uterine fibroids, primary and secondary tumour involvement of skeletal bones [15-20].

High-intensity ultrasound acts upon the tissues by warming them up. It penetrates through healthy tissues, causing a short-term, on the order of one second, temperature increase at the focal point by absorbing ultrasound waves and cavitation, sufficient for the development of coagulation necrosis [13]. In other words, the essence of such technologies using high intensity focused ultrasound (HIFU) is that the energy of the ultrasound beam penetrating into the human body from an external source is absorbed in the area of the affected organ, resulting in thermal denaturation [14]. In summary, without compromising the integrity of the surrounding tissue, high-intensity focused ultrasound allows to destroy of the tumour tissue, in other words to perform «non-invasive surgery».

To achieve this effect, sound waves with a frequency of 0.8–4 MHz and an intensity of 100–10000 W/cm$^2$ are used. The intensity of ultrasound used for therapeutic purposes is categorised into high (1000–10000 W/cm$^2$), medium and low (< 3 W/cm$^2$). As a comparison, in ultrasound diagnostics, the tissue exposure power ranges from 0.004 to 7.5 W/cm$^2$ [20, 21].

At the IV International Symposium dedicated to focused ultrasound in 2014, two cases were presented involving the use of the ExAblate Neuro 4000 device for ultrasound-guided thermodestruction of intracranial neoplasms – glioblastoma (in the thalamus region) and metastasis. In both observations, MRI after the procedure demonstrated partial destruction of tumour tissue [22]. Attempts to use brain tumour ablation in the clinic were reported earlier, in 1996–2010 [23], but these were only single cases or phase I trials on series of up to 15 patients, and these studies did not provide sufficient data concerning long-term follow-up, in particular, the dynamics of tumour size. As will be demonstrated below, the main focus of subsequent studies in this area has been focused on the application of low-power FUS rather than ablation.

As ablation with high-intensity ultrasound has been applied in several areas of oncology, certain disadvantages have been revealed, such as the risk of skin and bone burns in the path of the ultrasound wave to the focus, the difficulty in visualising the irradiated areas in real time using diagnostic ultrasound machines, and the possibility of damage to the bone and vessels around the irradiated area as a result of heat diffusion from the focus area. Consequently, alternative methods of mechanical tissue destruction, or histotripsy, have been proposed that overcome these disadvantages and also offer some other advantages. One of these methods is the so-called boiling histotripsy. A.P. Duryea, et al. [24] provide the following definition: histotripsy is a technology of pulsating focused ultrasound, when the initiation and control of acoustic cavitation allow for precise mechanical fragmentation of tissues. High-intensity ultrasound pulses of millisecond duration are propagated from the transmitter and focused inside a specific organ. Due to nonlinear acoustic effects, the wave profile becomes distorted as it propagates away from the transmitter, resulting in a shock front at the focus at each wave period. Absorption of the energy of such a nonlinearly distorted wave with a shock front results in rapid localised heating of the tissue at the focus, leading to its boiling during each pulse. This produces a vapour cavity measuring millimetres in size, noticeably larger than the volume of the superheated tissue area. The interaction of the shock wave with this cavity leads to the formation of an acoustic fountain and, consequently, to the fragmentation of the tissue into small fragments of subcellular dimensions. That is, it is the «fountain» that has the main damaging effect, and heating plays the role of only a «trigger», while eliminating the risk of damage to healthy tissues in the path of ultrasound [15]. As a result of the appearance of vapour cavities, the ablation site is easily visualised with ultrasound as a hyperechogenic area.

There have been sporadic studies regarding the use of histotripsy in animals to date. For instance, the experiments with pigs managed to create necrosis zones in the cerebral cortex up to 1 cm in size without significant complications [25]. No significant complications were revealed in histotripsy of a mouse model of gliomas in the work of S.W. Choi, et al. [26]. Histotripsy using focused ultrasound in mice with glioblastomas leads to the release of tumour antigens and increases the number of immune cells in its microenvironment; in addition, in mouse models, histotripsy of liver, kidney, and pancreatic cancers, as well as neuroblastoma and melanoma, leads to a significant increase in animal survival [26, 27]. Clinical trials of histotripsy in brain tumours have not yet been conducted.

A relatively low ultrasound power is not used for ablation, but for implementation of various ways to improve drug delivery to the tumour zone [17, 21].

## INCREASED BLOOD-BRAIN BARRIER PERMEABILITY

The presence of the blood-brain barrier, the function of which is to protect the brain from toxic substances, is considered as one of the reasons for the low efficacy of chemotherapy for brain high grade gliomas [28]. The BBB is formed by endotheliocytes, processes of astrocytes, pericytes, neurons, microglia macrophages, in addition, it includes extracellular matrix and glycocalyx [16, 29, 30].

It is known that the BBB is sometimes disrupted in glioblastomas (in the so-called «leaky» areas), as evidenced by the areas of contrast agent accumulation during MRI; however, in other parts of the tumour the BBB remains intact [31, 32]. This «diversity» of BBB permeability in glioblastomas is favoured by a variety of angiogenesis mechanisms [31]. The BBB within the tumour prevents most antitumour drugs penetration. Specifically, in the work of J. Portnow, et al. [33] has been demonstrated that in the brain of patients with glioblastoma or metastases of malignant tumours the average concentration of Temozolomide after its oral administration is approximately 5 times lower than in plasma.

One exception is Erlotinib (Tarceva), a HER1/EGRF epidermal growth factor receptor tyrosine kinase inhibitor used to treat brain metastases in non-small cell lung cancer. This preparation has a number of essential properties: it is fat soluble; it has no charge; its molecule is less than 500 D; there is no rapid elimination of this drug from the CNS [16]. Surgery and radiotherapy contribute to BBB disruption, but apparently this effect in malignant gliomas is not sufficient, considering the low efficacy of most chemopreventive agents [16]. Obviously, direct injection of an antitumour drug into

the tumour or intrathecal injections could be considered to overcome the BBB, but these techniques are associated with neurotoxicity and it is difficult to ensure prolonged exposure of the drug to the tumour tissue [34].

In view of the above problems, the technique of applying low-intensity focused ultrasound (LFUS) was established. Preclinical and clinical studies have revealed that this method can temporarily (for 24–72 hours) open the BBB without tissue damage, facilitating the penetration of antitumour drugs into the glioma area, and repeated sessions are possible. This technique involves the use of low-power ultrasound waves combined with intravenously administered microbubbles. When microbubbles travelling through the bloodstream are exposed to LFUS, they begin to periodically expand and contract. This process is called stable cavitation, it has a mechanical effect over the vascular walls, leading to reorganization tight junction proteins and increased drug penetration through the BBB. In this case, due to the mentioned cavitation phenomenon, less ultrasound power is required to disrupt the BBB, which ensures its safety, which has been evidenced in a number of experiments involving small and large animals. At the same time, LFUS slows down the elimination of anticancer drugs from brain tissue [16, 33, 35]. Besides, temporary disruption of the BBB during LFUS exposure can cause an acute inflammatory response leading to activation of immune system elements in the glioblastoma microenvironment [34].

In clinical studies, T1-weighted and dynamic MRI with contrast are used to assess the degree of BBB opening. It remains to be elaborated, however, how to confirm the increased drug accumulation in the tumour when LFUS is used locally [14].

In a review by J.W. Roberts, et al. [17] there have been 10 recently completed or currently ongoing clinical trials concerning BBB opening by ultrasound in the treatment of glioblastoma in adult patients, which are conducted in a number of countries: USA, Canada, South Korea, Taiwan, France, Switzerland, Belgium (NCT03712293, NCT03744026, NCT04417088, NCT04446416, etc.). This technique is used in them to improve the efficacy of the following drugs: carboplatin, temozolomide, pacitaxel, bevacizumab. One of these studies is NCT04614493 (France). In this phase I study of 19 patients with recurrent glioblastoma, ultrasound was delivered by an implantable device (SonoCloud-1; CarThera, France) before intravenous carboplatin administration.

In those 11 patients who achieved clear BBB disruption according to MRI, survival rates were slightly higher than in those with no or minor disruption: overall survival was 12.9 and 8.6 months, and progression-free survival was 4.1 and 2.7 months, respectively. In general, the FUS procedure (so-called sonification) was tolerated satisfactorily; 2 cases of swelling in the tumour area were observed, the manifestations of which were controlled with steroids [35]. The results of the other mentioned studies have not yet been published. Apart from that, preclinical testing of the effect of low-intensity FUS aimed at increasing BBB permeability in midline gliomas for drugs from the group of small molecules and monoclonal antibodies is being carried out at the Princess Maxima Centre for Children's Oncology (Utrecht, the Netherlands) [36]. In this group's study involving a mouse model of pontine glioma, a more than 5-fold increase in Olaparib bioavailability due to the opening of the BBB by means of FUS was revealed, which slowed tumour development during radiotherapy, although it did not lead to increased survival of the animals. The authors conclude that preclinical studies should be continued [37].

## SELECTIVE ULTRASOUND EFFECTS ON TUMOUR CELLS

Undoubtedly of interest is the work of D.R. Mittelstein, et al. [38], conducted *in vitro* using cellular models of breast cancer, colon cancer and leukaemia, which demonstrated that the use of low-intensity ultrasound < 5 W/cm$^2$, with a frequency of 0.5–0.67 MHz, pulse duration > 20 ms leads to selective damage to the cells of these malignant tumours without significant effect on healthy immune cells or erythrocytes. Physical experiments have revealed that acoustic standing waves and cavitation lead to cytoskeletal disruption, increased expression of apoptosis markers and death of tumour cells. The selectivity of the effect against the tumour cells, according to the authors, advantageously distinguishes this technique from ablation with high-intensity ultrasound. The methodology needs further validation.

## INCREASING THE EFFECT OF RADIATION THERAPY USING FOCUSED ULTRASOUND

Moderate hyperthermia of tissues under the influence of FUS can lead to an increase in their oxygenation and perfusion, thus increasing their sensitivity to radiotherapy [39, 40]. The world's first clinical study involving the use of FUS for radiosensitisation in glioblastoma is currently being conducted in Taiwan [17].

## SONODYNAMIC THERAPY

So-called sonodynamic therapy (SDT) is now being developed, in which FUS locally converts a substance inactive against the tumour into an antitumour drug. As a sonosensitizing agent, for example, 5-ALA and Fluoroscein are used. These drugs selectively accumulate in tumours such as glioblastoma and are now being used to improve tumour imaging during neurosurgery as they are activated not only by sound but also by light. Ultrasound by not quite clear mechanisms, interacting with these drugs, leads to the formation of reactive oxygen species that cause apoptosis. Two

clinical studies (one in the USA, one in Italy) concerning the use of sonodynamic therapy in malignant gliomas are now being conducted [17]. Most accredited theories include the effects of cavitation, generation of reactive oxygen intermediates (ROIs), induction of apoptosis in cancer cells, improvement of anti-tumour immunity, inhibition of angiogenesis and induction of hyperthermia [41].

**1. Cavitation effect**

The cavitation mechanism involves the formation and expansion of microbubbles filled with gas or liquid medium. Bubbles are usually formed from gases dissolved in the medium or from pre-existing nuclei, such as microbubbles injected as ultrasound contrast agents. The negative «rarefied» components of ultrasonic waves allow the expansion of small stabilised gas-filled «cavities» or bubbles within a liquid medium [42]. Under the influence of ultrasound pressure, these bubbles commence to oscillate, causing vibrations of the cell membrane or, if higher intensity ultrasound is applied, strong shock waves, compressing and thereby causing mechanical damage to the surrounding tissues. This phenomenon favours the breakdown of water molecules and the subsequent formation of hydroxyl radicals and hydrogen atoms

**2. Reactive oxygen intermediates generation**

The scientific basis of SDT relies heavily over the generation of ROIs through the simultaneous combination of low intensity ultrasound, molecular oxygen and sensitising drug [42]. The generation of ROIs in SDT appears to be closely related to the cavitation effect: the maximum expansion of gas bubbles and the subsequent rapid implosion release significant energy, leading to an increase in temperature and pressure in the surrounding microenvironment. It has been suggested that these extreme temperatures and pressures at the time of explosion act as a «sonicochemical» reactor capable of generating ROIs in the presence of water and oxygen; these unstable molecules, if formed intracellularly, can exert strong cytotoxic effects such as oxidative stress, DNA damage and apoptosis, and can induce lipid peroxidation if formed near the cell membrane. ROI formation in this process is mainly attributed to sonoluminescence and pyrolysis mechanisms.

**3. Induction of apoptosis in cancer cells**

Under normal conditions, cells are able to clear a certain number of ROIs as they are normally produced in the body during respiration; however, during SDT, excess ROIs are generated that cannot be immediately excreted and cause oxidative stress within the cells. Oxidative stress affects mitochondrial membrane potential, which may eventually lead to apoptosis [42].

**4. Improved anti-tumour immunity**

Generally, the effects of SDT by targeting the tumour microenvironment and tumour cells (both immune infiltrating and tumour cells) may increase tumour immunogenicity.

**5. Angiogenesis restriction**

Even if this mechanism is still not evident, the anti-angiogenic effects of SDT with 5-ALK were observed by V Choi, et al. both *in vitro* and *in vivo*; in particular, they observed that SDT using low-intensity ultrasound significantly inhibited endothelial cell proliferation and migration *in vitro*, as well as the ability to form capillary networks; consequently, in an *in vivo* study on a rodent model of human tongue cancer xenograft they showed that the expression of vascular endothelial growth factor, a critical proangiogenic factor, was significantly reduced after SDT treatment compared to subjects receiving only ultrasound or a control group [42, 43].

**6. Hyperthermia induction**

In some preliminary studies, ultrasound-induced hyperthermia has been demonstrated to enhance the effect of SDT, although the exact mechanism of this effect is still not evident [41].

## FOCUSED ULTRASOUND AND LIQUID BIOPSY

Y. Meng, et al. [44] after exposure to low-power FUS at the tumour areas in 9 patients with glioblastomas observed an increase in the blood of brain and tumour biomarkers, in particular, extracellular circulating DNA, the methylation profile of which indicated its possible origin from the tumour. The authors attribute this effect to the temporary opening of the BBB as affected by ultrasound. Previously, similar effects were observed in animal experiments [45, 46]. Proceeding from these data, it can be concluded that FUS is a promising method for increasing the informativeness of liquid biopsy in malignant gliomas of the brain.

## CONCLUSION

These literature data evidence that focused ultrasound of different intensities is considered by researchers in a number of countries as one of the promising ways to improve the effectiveness of treatment in oncology, particularly in brain tumours. However, it should be emphasised that studies in this area have so far been conducted mainly in the form of basic physical research and experiments on cellular models and animals. Clinical trials are still very few and have not exceeded in general the Phase I. Nevertheless, one cannot ignore the fact that in our country there is a well-defined basis for conducting studies related to the application of FUS in clinical terms. At the same time, the substantial experience of a number of Russian clinics in the treatment of malignant tumours of the CNS using modern conventional high-tech antitumour methods both in adults

and children, as well as the availability of modern equipment for MRI and ultrasound diagnostics, including liquid biopsy, and, along with this, the availability of original developments of Russian physicists allow not only to continue, but also to intensify the studies aimed at a deep understanding of the exact mechanisms involved, in particular, in focused ultrasonic disruption of the BBB.

Studies involving the use of sonosensitisers and ultrasound for sonodynamic treatment of gliomas, however, are currently at a very early stage. That said, before FUS becomes a routine treatment option for CNS tumours, studies are needed to confirm the sonodynamic effect in other preclinical glioma models besides the most commonly used rat C6 glioma cell line. More importantly, further studies to determine the optimal ultrasound wave parameters required for induction of sonodynamic effects in malignant cells without causing damage to healthy brain tissue are also a priority. Overall, the results obtained in such experiments may be invaluable for planning further *in vivo* preclinical studies and, in the future, clinical studies.

Concurrently, the preliminary clinical data published to date appear to offer the exciting prospect of a new method for the specific treatment of glioma that may be selective to malignant cells and at the same time practically non-invasive, which will allow its future use primarily in paediatric patients, as well as its repeated application in case of disease recurrence, especially after previous radiotherapy.

**Conflict of interest**

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# POST-CASTRATION SYNDROME: RELEVANCE, IMPACT ON QUALITY OF LIFE, METHODS OF CORRECTION

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## ABSTRACT

*Aspects of modern medicine cover a huge number of diseases, including post-castration syndrome, which occurs as a result of endocrine testicular dysfunction in men. The relevance of this condition is great, since it is caused by many reasons and is the basis for an inferior life in men. The review shows the impact of this disease on the quality of life and the methods for its treatment. When writing this review, we used data on castration methods and its correction based on materials published in the eLibrary and PubMed databases. The search was carried out using the keywords: "castration", "post-castration syndrome", "prostate cancer". With castration, there is a decrease in serum testosterone levels, as androgens stimulate the growth of prostate cancer. Today, hormone therapy is an alternative to castration in the treatment of prostate cancer. Surgical castration is the gold standard; it can suppress tumor cell proliferation and induce tumor apoptosis, but it causes significant impairment of quality of life. The article presents characteristics of medications, indications, contraindications and side effects of hormone therapy. The quality of life of men with testosterone deficiency is clinically associated with the development of metabolic syndrome, manifested by obesity, hepatic steatosis and type 2 diabetes mellitus. Numerous studies by domestic and foreign scientists confirm the effect of castration in men on their body, which increases the risk of stroke, depression, cognitive disorders and Alzheimer's disease. Thus, despite the significant advances of modern medicine in the treatment of malignant diseases of prostate, post-castration syndrome remains a completely unexplored problem, which indicates the need for further study and the development of effective therapy.*

***Key words:*** *prostate cancer, testicular cancer, orchiectomy, post-castration syndrome, castration*

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# ПОСТКАСТРАЦИОННЫЙ СИНДРОМ: АКТУАЛЬНОСТЬ, ВЛИЯНИЕ НА КАЧЕСТВО ЖИЗНИ, МЕТОДЫ КОРРЕКЦИИ

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## РЕЗЮМЕ

*Аспекты современной медицины охватывают огромное количество заболеваний, в том числе посткастрационный синдром, возникающий в результате эндокринной дисфункции яичек у мужчин. Актуальность данного состояния велика, так как она возникает вследствие многих причин и является основанием для неполноценной жизни у мужчин. В обзоре показано влияние данного заболевания на качество жизни и методы его терапии. При написании данного обзора были использованы данные о методах кастрации и её коррекции по материалам, опубликованным в базах eLibrary и PubMed. Поиск проводился по ключевым словам: «кастрация», «посткастрационный синдром», «рак предстательной железы». При кастрации наблюдается снижение уровня тестостерона в сыворотке, так как андрогены стимулируют рост рака предстательной железы. На сегодня гормональная терапия является альтернативой кастрации при лечении пациентов с раком предстательной железы. Хирургическая кастрация является «золотым стандартом»; с её помощью можно подавить пролиферацию опухолевых клеток и индуцировать апоптоз опухоли, но она вызывает значительные нарушения качества жизни. В материале представлены характеристика лекарственных препаратов, показания, противопоказания и побочные эффекты от проводимой гормональной терапии. Качество жизни мужчин с дефицитом тестостерона клинически связано с развитием метаболического синдрома, проявляющимся ожирением, стеатозом печени и сахарным диабетом 2-го типа. Многочисленные исследования отечественных и зарубежных учёных подтверждают влияние кастрации у мужчин на организм, при котором повышается риск развития инсульта, депрессии, когнитивных расстройств и болезни Альцгеймера. Таким образом, несмотря на значительные успехи современной медицины в терапии злокачественных заболеваний предстательной железы посткастрационный синдром остаётся до конца не изученной проблемой, что свидетельствует о необходимости дальнейшего изучения и разработки эффективных средств терапии.*

***Ключевые слова:*** *рак предстательной железы, рак яичка, орхиэктомия, посткастрационный синдром, кастрация*

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## INTRODUCTION

Post-castration syndrome (PCS) in men is a symptom complex characterised by endocrine, metabolic and neuropsychiatric disorders that develops as a consequence of endocrine dysfunction of the testicles as a result of surgery, trauma, disease, radiation and tissue destruction after infectious diseases. Cancer ranks second in the mortality structure, behind cardiovascular diseases, and is responsible for up to 11 % of all deaths worldwide. Each year, 18.1 million people get cancer and 9.6 million of them die; thus, 27,000 people die of cancer per day. According to Rosstat (Russian Federal State Statistics Service), in 2018, 41.1 men per 1,000 people aged 16 to 59 years died from malignant neoplasms (96.2 men per 100,000 population); despite a slight decrease in 2019 (respectively to 38.8 and 91.9 men), in general, the number of cases of this pathology does not tend to decrease [1, 2].

All endocrine glands are susceptible to neoplastic growth, but the health effects of these neoplasms are diverse. Pituitary tumors are widespread and the vast majority of cases are benign, but are characterized by a wide range of different effects on the body. Prolactinomas, by causing an increase in prolactin concentration in the blood, exert their influence on the function of the sexual glands through the hypothalamus, contributing to the development of hypogonadotropic hypogonadism. Microprolactinomas are most common in women, causing infertility and menstrual irregularities (secondary amenorrhea), while in men, on the contrary, macroprolactinoma is more frequently observed [3].

The prevalence of urogenital injuries in peacetime is 1–3.5 % of the total number of cases, of which 35–60 % is injuries involving the kidney and 30–41 % involve the scrotum and other parts of the genital organs. A significant proportion of the PCS patient population is prostate cancer (PC), which is the leading cause of death in men. Globally, 1.6 million men are diagnosed with PC each year with 366,000 men (22.88 %) dying. Numerous literature data reveal a significant reduction in the quality of life of men, indicating the need for ongoing rehabilitation in them. In the structure of cancer morbidity in men in the Russian Federation PC occupies the 4$^{th}$ place, and over the last 10 years the incidence has increased more than 2-fold, and its growth rate is currently the highest among all cancer diseases in males [4].

PC has the highest prevalence in developed countries; for example, the probability of diagnosis at age 79 years is 1:47 in countries with a low and/or medium socio-demographic index. In the US, PC is the leading cause of PCS morbidity and mortality; an estimated 180,890 new cases were diagnosed in 2016. The ratio of morbidity to mortality varies considerably around the world, with the highest ratio in North America (10:1), a lower ratio in Australia (2:1), and almost the same in some Caribbean countries and parts of Africa (1.2:1). The ongoing radical treatment of PC can have a negative impact and cause not only disorders of urinary function, bowel function, sexual dysfunction, but also quality of life, which makes specialists think about the search for new methods of therapy.

Despite its high incidence, prevalence and duration, this type of cancer has the highest 5-year survival rate, accounting for 25 % of all cases.

Other reasons for the development of PCS are testicular trauma caused by sex reassignment surgeries, congenital malformations and anomalies, other oncological, somatic diseases and complications from surgical interventions. The use of Student's criterion is recommended to estimate the incidence and statistical treatment of the injury number in the United States, which allows for the collection of clinical and anamnestic patient data that includes age, gender, and comorbidities at the pre-hospital stage. Pre-hospital comorbidities included cardiovascular insufficiency, arterial hypertension, peripheral vascular disease, myocardial infarction, bad habits (smoking, alcohol), renal failure, pulmonary disease and cerebral circulatory disorders. Injury profile information included a scale to assess the severity and mechanism of injury.

Any injuries to the kidneys, urinary bladder, urethra, ureter, adrenal glands, penis, pelvis, spine and head injuries were also included in the study. An analysis of scrotal and testicular injuries in US patients revealed that among 8030 patients, only 19 (0.23 %) had scrotal/testicular injuries, of which only 8 (44.6 %) cases were due to blunt trauma. In this case, the penetrating mechanism of injury occurred in 50.5 per cent of cases, and the most frequent cause (75.8 %) was assault with a firearm. The mean age of the patients was 31 ± 3.6 years. The majority of patients (74.5 %) had an isolated injury to the scrotum or testis, which required operative treatment to repair the rupture in 48.3 % of cases (37.3 %). One of the causes of testicular injuries was in cases where patients were involved in motorbike accidents; in this group, scrotal/testicular surgery was performed in half of the cases (48.3 %), but the most common surgical procedure was correction of scrotal or testicular rupture (in 37.3 % of cases) followed by unilateral orchiectomy in 23.4 % of cases [5].

Abroad, due to certain psychological and physiological factors, surgeries are often performed for cosmetic purposes: for example, sex reassignment surgery for transgender women. Gender dysphoria is a condition in which a person's sex assigned at birth and the gender with which they identify are not compatible. The American Society of Plastic Surgery's annual report of specialists revealed that 1,759 transfeminine and 1,497 transmasculine surgeries were performed among patients who underwent gender confirmation in 2016, an increase of 27 % and 10 %, respectively, compared to 2015. A recent survey of nearly 28,000 transgender people revealed that among people who were assigned female sex at birth, 21 % had undergone breast reconstruction surgery, 8 % had a hysterectomy, 1 % had a metoidioplasty, and 1 % had a phalloplasty. Among those classified as male at birth, 10 % underwent vaginoplasty

or labioplasty, 9 % underwent orchiectomy, 8 % underwent augmentation mammoplasty, and 6 % underwent facial surgery [6-8].

It is not uncommon to develop PCS with testicular torsion, a situation that is an acute urological emergency affecting 1 in 4,000 men under the age of 25. It results from the rotation of the spermatic cord, where the rotation and subsequent narrowing of the artery leads to ischaemia, causing damage to the testicular tissue, which is also a condition requiring emergency surgical care. Ultrasound testicular sonography in this case is the basis for assessing blood flow and the severity of vascular changes in order to decide whether to preserve the organ [9].

Surgery for congenital anomalies and malformations with undescended testis (ICD-10: Q53.9) is a situation in which orchiectomy may also be required; it occurs when the testicles are located in the abdominal cavity and need to be surgically transferred to the scrotum. If the damage is irreparable, the surgeon may extirpate one or both testicles, so a thorough chiropractic examination is necessary to rule out medical errors. To achieve this, after laying the patient down, warm fingers should be used to palpate the organ from the projection of the inguinal canal to the pubis in the supine position of the patient. The groin, scrotum, inner thigh, femur, perineum and penile region should also be palpated to reveal an ectopic testis. Visualising studies (ultrasound) may influence the treatment tactics and diagnostic laparoscopy is unavoidable to independently evaluate the results [10, 11].

Hormone therapy (HT) has significant advantages over surgical castration (SC) as it can be administered in short or long courses, but it also has adverse effects on the body – it can cause decreased muscle mass, sexual dysfunction, physical weakness, psychological trauma and reduced quality of life (QOL). Adverse metabolic effects of HT are often associated with an increased risk of cardiovascular and neuropsychiatric disease. Immediate symptoms in locally advanced and/or metastatic cancer include: pain, hypercalcaemia, spinal cord compression and pathological bone fractures, which also have a negative impact on general health. Therefore, conservative therapy in patients with PC can improve QOL, preserve and restore urinary, defecation and genital functions [2].

## MATERIALS AND METHODS

Data on PCS, methods of its correction published in eLibrary, PubMed databases were used to write this review. The search was performed by keywords: "castration", "post-castration syndrome", "prostate cancer". As a result of detailed source validation, 60 articles included in the review were selected directly for citation to be used for the study.

Castration is defined as "the deprivation of (male animal or human) testicles" and has distant roots. Surgical castration by orchiectomy (removal of the testicles) was often performed in ancient China. A study of eunuchs in China revealed that castration resulted in 21 of 26 men having little or no prostate gland palpability, suggesting that androgens need to be constantly present throughout of life for the gland to function. Disappearance (reduction in size) of the prostate can be functional (after castration) and age-related, when there is a natural shrinkage of the testicles. Castration today can be performed surgically and chemically [12].

Lack of androgenic stimulation of tumour cells by surgical or pharmacological castration leads to suppression of their proliferation and induces tumour apoptosis. Surgical castration is the «gold standard» against which all other HT methods are compared; removal of the testicles after 24 hours reduces serum testosterone levels to minimal levels. Today orchiectomy is no longer the main method of treatment, mainly because of public opinion, which considers this method barbaric, capable not only of changing the appearance, but also of having a pronounced psychological impact since the procedure is irreversible. The only advantages of this method are simplicity of execution, cheapness and lack of complications, but this method is not popular among patients because of the pronounced negative psychological effect of the operation and irreversibility of the process. Pharmacological castration, therapy with luteinising hormone-releasing hormone (LHRH) agonists – analogues of natural human LHRH that achieve reversible medical castration and antiandrogens, which have the ability to produce maximal androgen blockade and intermittent androgen suppression, are increasingly used for this purpose. However, despite the variety of existing approaches and the vast clinical experience with HT, no «ideal» method of androgen deprivation has yet been found. Recently, new groups of drugs for HT of PC – LHRH antagonists – have appeared on the pharmacological market [13-15]. With this type of castration, there is a decrease in serum testosterone levels, as androgens are known to stimulate prostate cancer growth; therefore, HT can be used to treat patients even with late diagnosed PC [16, 17].

One type of HT is androgen-deprivation therapy (ADT); in men with metastatic PC, it induces an antitumour response and "improves malignancy-associated bone metabolic abnormalities". ADT can be prescribed for varying lengths of time and can be combined with remote radiation therapy or brachytherapy (interstitial irradiation). The course of HT usually depends on the disease, side effects, complications and contraindications accompanying the therapy, ability to alter testosterone and estrogen levels in men. Several different types of HT exist today, consisting of chemical and surgical means. Chemical castration is performed by three groups of pharmacological agents [18, 19].

The first type includes LHRH agonists or antagonists; LHRH agonists sometimes can cause "testosterone flares" that can be avoided with antagonists. LHRH

agonists include leuprorelin, goserelin, and triptorelin; LHRH antagonists include degarelix, CYP17 inhibitors, and abiraterone [20].

The second group includes antiandrogens, which are involved in blocking the effects of androgens directly on cells, usually through blockade of the androgen receptor itself. These include flutamide, bicalutamide, nilutamide, enzalutamide, apalutamide and darolutamide. These oral medications are divided according to their chemical structure into steroidal (cyproterone, megestrol and medroxyprogesterone) and non-steroidal or pure (nilutamide, flutamide and bicalutamide) [21, 22].

The third group includes preparations of female sex hormones – estrogens. Estrogens act through a feedback mechanism, shutting down the production of androgens and indirectly reducing testosterone levels. The main side effect associated with estrogen administration is that its high activity (due to the presence of estrogen receptors on platelets) can lead to serious or even fatal venous thrombosis (VTE) [21].

The main advantages of ADT are that the therapy results with a reduction in testosterone levels that is rapid and effective, non-surgical, reversible and has the option for oral or subcutaneous administration, unlike current chemotherapy drugs administered intravenously. However, the side effects resulting from HT are significant and can often lead patients to discontinue its use or use it intermittently. The main limitations are hot flashes, loss of libido, fatigue, osteoporosis, erectile dysfunction and more. One of the biggest limitations to the use of HT is the fact that disease recurrence with the development of castration-resistant prostate cancer (CRPC) is often observed after HT [22, 23].

Long-acting LHRH analogues have become the main form of HT in PCS. The drugs are synthetic analogues of LHRH, mainly administered as depot injections once every 1, 2, 3, 6 or 12 months. After a single dose, they stimulate LHRH receptors, causing a transient increase in LH and follicle-stimulating hormone (FSH) release. This in turn causes an increase in testosterone synthesis ("waves" of testosterone or a "flash" effect) that begins 2–3 days after the first injection and lasts for about one week. The "flash" effect can lead to potentially deleterious effects and is manifested by increased bone pain, acutely developing infravesical obstruction, postrenal renal failure, spinal cord compression, and fatal cardiovascular complications associated with a state of hypercoagulability. The risk of developing clinical "flash" events is increased in patients with symptomatic large-volume bone metastases. Concomitant therapy with antiandrogens reduces the frequency of clinical "flashes" but does not completely exclude the possibility of their occurrence [24, 25].

**LHRH antagonists** are able to bind immediately to LHRH receptors in the anterior lobe of the pituitary gland. This results in a rapid decrease in LH, FSH and testosterone levels without the development of "flashes", which improves therapy outcomes and compliance on the part of patients. The lack of long-acting depot forms is considered a disadvantage and a practical limitation to the use of the drugs, as only monthly administration formulations are currently available [26].

One of the drugs in this group is the LHRH antagonist Degarelix, which is administered subcutaneously once a month. The standard dose of Degarelix is 240 mg for the first month, followed by 80 mg monthly thereafter. The indication for this drug is advanced hormone-dependent PC; side effects include decreased libido, headaches, depression, nausea, diarrhoea, muscle pain, increased sweating, increased urination, and anaemia, which should be considered when prescribing the drug [27].

The group of steriod inhibitors of CYP17 enzyme includes the drug Abiraterone, which is used for the treatment of metastatic castration-resistant prostate cancer (in combination with prednisolone). Side effects include urinary tract infections, hepatotoxicity, arterial hypertension, dyspepsia, and peripheral oedema [28].

**Steroidal antiandrogens** are synthetic derivatives of hydroxyprogesterone. Side effects develop when prescribing drugs of this group develop secondary to a decrease in testosterone levels (gynecomastia is quite rare), and non-pharmacologic include cardiovascular complications (4–40 % for cyproterone) and hepatotoxicity. Ciproterone is usually prescribed in a regimen of two or three times 100 mg; the indication for its prescription is the correction of pathological abnormalities in the field of sexual behavior (if it is necessary to reduce sexual activity), metastasizing or inoperable cancer. Side effects include gynecomastia, weight change, depression, fatigue, cough, and dyspnea [29, 30].

**Nonsteroidal antiandrogens** do not inhibit testosterone secretion; therefore, libido, general physical well-being, and bone mineral density (BMD) are preserved during antiandrogen therapy [31, 32]. However, nonpharmacologic side effects may be observed when prescribing drugs of this group: visual disturbances (delayed adaptation to darkness), alcohol intolerance, nausea, hepatotoxicity and interstitial pneumonia (sometimes fatal) and headache [33, 34].

**Estrogens** reduce testosterone levels, but their use causes severe side effects in the form of thromboembolic complications even at the lowest possible doses, so this group is not considered for therapy [35, 36].

The assessment of QOL in men with testosterone deficiency revealed a clinical association with the formation of metabolic syndrome, which is manifested by the development of obesity, liver steatosis and type 2 diabetes mellitus. Evidence for this comes from experimental studies on male rats, confirming the effects of testosterone deficiency caused by castration and the development of body obesity. Numerous studies by domestic and foreign scientists confirm the effect of castration in men on their body, which increases the risk of stroke, depression, cognitive disorders and Alzheimer's disease. In contrast, men who are castrated before puberty retain prepubertal features

such as a high voice and lack of facial vegetation; when castration is performed after puberty, facial vegetation and a low voice are retained, but men experience physical changes such as loss of body hair, some gynaecomastia and reduced penile size. Side effects of castration can be observed in long-term use of LHRH agonist, when in addition to feminising signs there is weight gain of up to 10 %, mainly due to fat in the abdomen and thighs, loss of muscle mass by 3–4 % on average, mild anaemia, osteoporosis, hot flashes. At the same time, more than 85 % of men with androgen deficiency report decreased libido and erectile dysfunction. In the differential diagnosis, depressive disorders in PC patients who have undergone SC and are deprived of androgen exposure are difficult to distinguish from those with ineffective cancer therapy and aging [37-40].

Depressive disorders in patients with cancer can have a range of negative effects, which include increased risk of suicide, reduced quality of life, reduced life expectancy, poor patient compliance and increased length of stay in oncology hospital. There have been several studies where it is found that single and unmarried men have a 65 % chance of committing suicide. And the probability of suicide is the highest in the group of patients diagnosed earlier, which is probably associated with an increase in the duration of the impact of risk on the patient's psyche. Other important factors influencing the frequency of depressive disorders include: somatic diseases depending on the degree of severity; complications associated with mono- or multi-organ lesions of organs and systems, side effects of drugs (cytostatics, hormonal agents, radiation therapy, etc.).

The stage of cancer has a significant impact on the frequency and severity of depressive disorders. Some of the risk factors for the development of depressive disorders are the severity and traumatic nature of the surgical procedure during surgical treatment. Most often it is represented by polymorphic fears and nosophobia formed against the background of persistent hypothymia (depression, pessimistic assessment of future prospects), which are closely related to the situation of cancer [41, 42].

Different changes in emotional behaviour have been reported in men and transsexuals who have transitioned from male to female. Despite gender reassignment, men have a difficult time dealing with the onset of changes in appearance that are not always appropriate and affect their ability to function sexually. Many studies show that the physical and psychological effects of ADT are severe, leaving patients powerless to cope with depression, cognitive impairment, and dramatically reduced QOL. With reduced libido, many subsequently refuse sex, love and physical contact from their partners. They are embarrassed by the changes in appearance, deeply worried and do not want to discuss this topic, even with doctors, which leads to frustration and depression in partners, and later - to the breakdown of marital relations [43-45].

At the same time, the clinic of PCS development varies significantly depending on age. When comparing patients castrated for PC and voluntary eunuchs, the latter reported higher levels of sexual interest despite being on average two decades younger than the cancer patients. From the perspective of both groups, their self-rated physical health was clearly higher than that of the PC patients, also helped by the fact that they were not burdened by a cancer diagnosis. Nevertheless, these data suggest that the biological factors mentioned - age, health, hormones, and psychological expectations - also affect sexuality in androgen-deprived men. That said, it is worth noting that eunuchs in history have had higher QOL, which depended on cultural norms, time and place of residence.

Voluntary eunuchs had higher levels of mental health and social interaction, with at least 49 % reporting major or minor depression prior to castration, and only 38 % reporting depression after castration, indicating stress. Studies conducted abroad confirm that only 17 % of eunuchs who underwent SC cite their desire to be a woman as the main reason, while 15 % stated that they used this method to give pleasure to their partner. Voluntary eunuchs have one thing in common with PC patients – they hide from prying eyes, and few are able to talk openly about their castrated status in everyday life. However, only 8 % of surgical eunuchs report that they have had this surgery performed against the opinion of their closest friends, relatives and family members. All this suggests that most castrated individuals conceal their morphological and emotional colouration for fear of humiliation in society [46-49].

Thus, despite significant advances in modern medicine in the therapy of malignant prostate cancer, post-castration syndrome remains an understudied problem. The analysis of the material indicates the need for further study and development of new quality drugs that can influence the course of the malignant process and at the same time have little effect on the quality of life. The development of drugs capable of medically correcting androgen deficiency in patients is important in order to eliminate possible physical and psychological changes, to improve the quality of life and health status of patients suffering from malignant disease.

**Conflict of interest**

The authors declare that the submitted article, its topic, subject and content do not involve competing interests.

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## OPHTHALMOLOGY

# VIRTUAL AND EXPERIMENTAL SCREENING OF NEW MELATONIN BIOISOSTERES FOR THE TREATMENT OF GLAUCOMA

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## ABSTRACT

***Background.*** *Melatonin is an endogenous regulator of intraocular pressure (IOP), but its effectiveness as a drug for glaucoma treatment is limited.*
***The aim of the study.*** *To develop and to validate a virtual screening method to identify bioisosteric analogs of melatonin that are promising for study as agents that reduce intraocular pressure.*
***Results.*** *A database containing structural and experimental affinity information for 48 individual reference compounds was created. Risk assessments for mutagenic, carcinogenic, irritant and reproductive toxicity were performed in DataWarrior based on substructural analysis and identification of fragments that are markers of relevant toxicity. A virtual screening of 2457 structures was carried out and 25 compounds from the selected ones were experimentally studied for their effect on intraocular pressure (IOP) in intact rats. 10 of the 25 prioritized compounds were found to significantly reduce IOP; compound RU-398 reduced IOP by 40 %, K-165 – by 40.9 %, and RU-615 reduced glaucoma by 33.3 %.*
***Conclusion.*** *The effectiveness of virtual screening after experimental validation was 40 %. The identified active compounds are promising for further study and development as the agents for the treatment of glaucoma.*

***Key words:*** *isostere, melatonin, glaucoma, intraocular pressure, virtual screening*

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# ВИРТУАЛЬНЫЙ И ЭКСПЕРИМЕНТАЛЬНЫЙ СКРИНИНГ НОВЫХ БИОИЗОСТЕРОВ МЕЛАТОНИНА ДЛЯ ЛЕЧЕНИЯ ГЛАУКОМЫ

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## РЕЗЮМЕ

***Обоснование.*** *Мелатонин является эндогенным регулятором внутриглазного давления (ВГД), но эффективность его применения в качестве лекарственного средства при глаукоме ограничена.*

***Цель исследования.*** *Разработка и валидация метода виртуального скрининга для выявления биоизостерических аналогов мелатонина, перспективных для изучения в качестве средств, снижающих внутриглазное давление.*

***Результаты.*** *Была создана база данных, содержащая информацию о структуре и экспериментальном сродстве 48 отдельных эталонных соединений. Оценка риска мутагенной, канцерогенной, раздражающей и репродуктивной токсичности была выполнена в DataWarrior на основе подструктурного анализа и идентификации фрагментов, которые являются маркерами соответствующей токсичности. Был проведён виртуальный скрининг 2457 структур и 25 соединений из числа отобранных были экспериментально изучены на предмет их влияния на ВГД у интактных крыс. Было обнаружено, что 10 из 25 приоритизированных соединений способны значимо снижать ВГД; соединение RU-398 снижало уровень ВГД на 40 %, K-165 – на 40,9 %, а RU-615 снижало глаукому на 33,3 %.*

***Заключение.*** *Результативность виртуального скрининга после экспериментальной валидации составила 40 %. Выявленные активные соединения являются перспективными для дальнейшего изучения и разработки в качестве средств для лечения глаукомы.*

***Ключевые слова:*** *изостер, мелатонин, глаукома, внутриглазное давление, виртуальный скрининг*

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## INTRODUCTION

Glaucoma is a multifactorial chronic progressive optical neuropathy characterised by damage to the optic nerve and retinal nerve fibre layer, which can lead to irreversible loss of peripheral or central vision [1]. Currently, glaucoma is a collective term for a group of neurodegenerative processes affecting the entire visual pathway, characterised by progressive, irreversible destruction and death of retinal ganglion cells [2].

One promising class to look for antiglaucoma drugs is melatonin analogues. Melatonin is the hormone responsible for regulating circadian and seasonal rhythms. This molecule was first discovered and described in the pineal gland, but it is now known to be synthesised in many tissues of the body, including the eye and ocular structures, particularly the retina, iris, ciliary body, lens and lacrimal gland, where it regulates important processes. Its effect on intraocular pressure (IOP) is mediated through $MT_1$, $MT_2$ and the putative melatonin receptor $MT_3$ located in the ciliary body, resulting in a decrease in chloride outflow from non-pigmented epithelial cells by increasing cyclic adenosine monophosphate. A decrease in this outflow causes a decrease in the production of aqueous moisture and finally a decrease in IOP [3].

The search for new melatonin analogues with greater metabolic stability and duration of action is an active area of ongoing study. Compounds of various structures with affinity for melatonin receptors have been described [4].

## THE AIM

To develop and to validate a virtual screening method to identify bioisosteric analogs of melatonin that are promising for study as agents that reduce intraocular pressure.

## METHODS

### Compliance with Ethical Standards

The experimental work was carried out in accordance with the requirements of GOST ISO/IEC 17025-2009, GOST R ISO 5725-2002 and the rules of laboratory practice in conducting preclinical studies in the Russian Federation in accordance with GOST R 33044–2014 'Principles of Good Laboratory Practice' and Order of the Ministry of Health of the Russian Federation № 199n dated April 1, 2016 «About the approval of the rules of good laboratory practice», in compliance with Directive 2010/63/EU of the European Parliament and Council of the European Union of September 22, 2010 concerning the protection of animals used for scientific purposes.

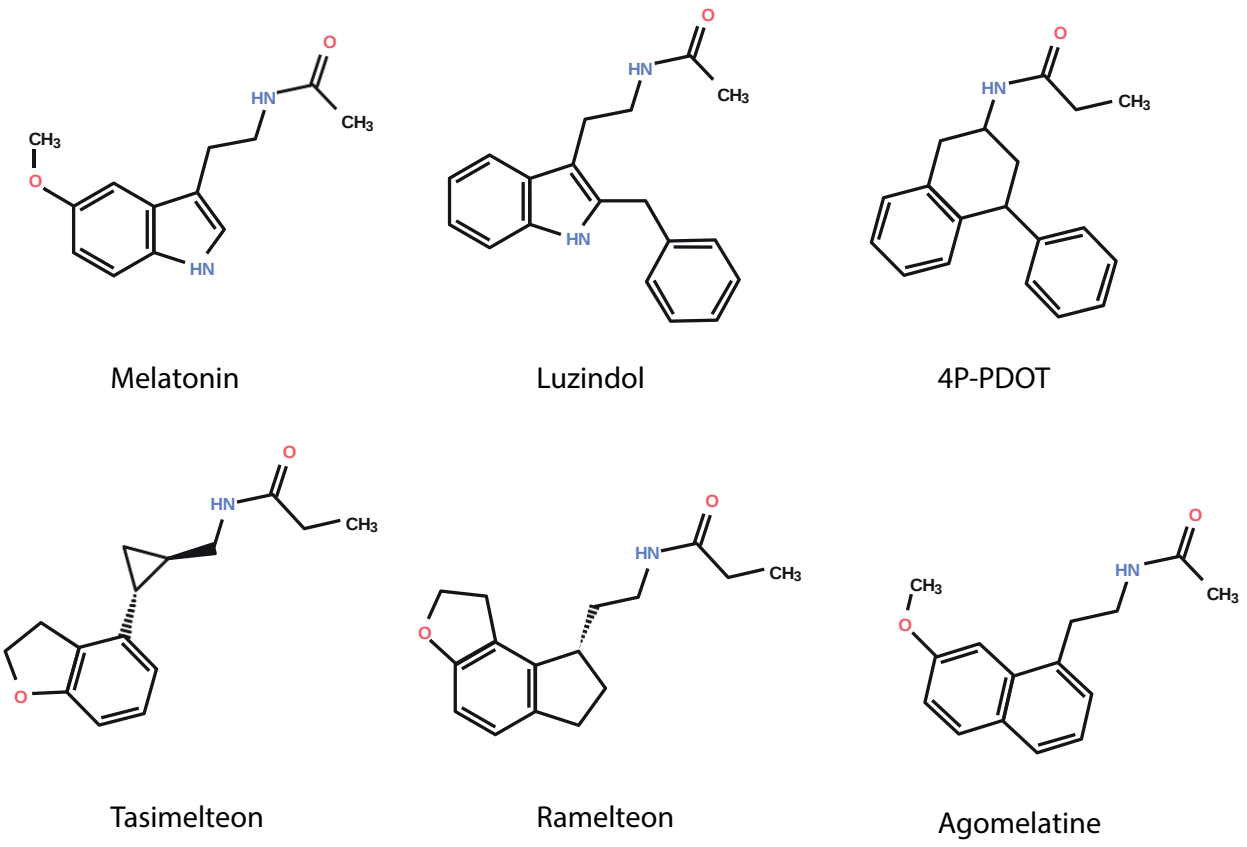



***FIG. 1.***
*Melatonin and some of its bioisosters*

### Data preparation

In the preparation of the structure database of compounds available for study, duplicates, salt residues were removed and structures were standardised. A database containing 2457 investigated compounds was created.

### Reference structures

Information from the IUPHAR pharmacological database was used as a source of validated ligands with affinity for human type 1 and type 2 melatonin receptors ($MT_1$ and $MT_2$) [5].

### Molecular properties calculation

For each compound, characteristics related to drug similarity were calculated, including molecular weight *M*, lipophilicity index *ClogP*, number of donors and acceptors of hydrogen bonds $H_{donors}$ and $H_{acceptors}$, number of halogen atoms $N_{halogens}$, number of heavy atoms $N_{heavy}$, polar surface area *PSA* (polar surface area). All the described calculations were performed using the OSIRIS DartaWarrior program (Idorsia Inc., Switzerland) [6].

### Assessment of substructural markers of specific toxicity

Risk assessment of mutagenic, carcinogenic, irritant properties and reproductive toxicity was performed in DataWarrior based on substructural analysis and identification of fragments known to be markers of relevant types of toxicity.

### *DrugScore* integral measure of drug eligibility

*DrugScore* is an integral index including substructural drug similarity, lipophilicity, solubility, molecular weight and risk of specific toxicity (carcinogenicity, mutagenicity, local irritant and reproductive toxicity). The calculation was performed according to [6].

### Calculation of corneal permeability to compounds

The calculation of the corneal permeability index for *LogPapp* compounds was performed according to two previously published QSAR models [7, 8] using Eqs:

$$LogPapp_1 = -4{,}002 - 0{,}169 \times (H_{acceptors} + H_{donors}) + 0{,}265 \times LogP;$$

$$LogPapp_2 = 4{,}6823 - 0{,}767 \times (Log(PSA)) - 0{,}1346 \times H_{donors} + 3{,}0024 \times N_{halogens}/N_{heavy};$$

$$LogPapp = (LogPapp_1 + LogPapp_2)/2.$$

### Molecular similarity calculation

DataWarrior *Flexophore* 3D pharmacophore descriptor was used as molecular descriptor for molecular similarity calculation. Computation of the *Flexophore* descriptor begins by creating a representative set of up to 250 conformers using a self-organisation-based algorithm to construct small rigid fragments of the molecule, which are then joined by considering the likely torsional angles. Atoms of the molecule that could potentially interact with the protein atoms in some way are then detected and classified. The extended MM2 atom typing is used to describe atoms as interaction points. The Tanimoto coefficient served as a quantitative metric of similarity between the studied compounds and the reference compounds. For each compound, the maximum revealed coefficient of molecular similarity according to the pharmacophore descriptor *Max* (*Flexophore*) was considered.

### Calculation of the overall prospectivity index

The integral prospectivity score of compound *F* was calculated based on *DrugScore*, calculated *LogPapp* corneal permeability and maximum pharmacophore similarity to *Max*(*Flexophore*) reference standards converted between 0 and 1 with the inflection point of the sigmoid curve at a given parameter boundary value and curve slope and parameter weight according to the table and formula (Table 2):

$$p = 1/[1 + e^{(a \times p + b)}].$$

### Intraocular pressure measurement

The study of the effect upon intraocular pressure was performed on mongrel intact rats of both sexes weighing 220–400 g, aged 2 months (Rappolovo husbandry, Leningrad region). All animals were divided into experimental and comparison drug groups with 6 animals in each group. At 9:00 am, baseline IOP in both eyes was measured in animals of all groups. The veterinary tonometer ICARE TonoVet (Finland) for early diagnosis of glaucoma in veterinary medicine was used to determine IOP [9, 10]. After the measurement, the animals of the experimental groups were instilled with 0.4 % aqueous solutions of the studied compounds in the volume of 50 µl into the right eye (test eye). Animals of the comparison drug groups had melatonin (Sigma, USA; pharmaceutical standard) instilled into the test eye. The left eye (control, collateral eye) served to determine the possible resorptive effect. Follow-up IOP measurements in the test and collateral eyes were performed after 60, 120, 180 min.

### Data analysis

Chemoinformatic calculations were performed in DataWarrior software (Idorsia Inc., Switzerland). The computational data processing was performed in RStudio 2022.07.1. Statistical processing of experimental data was performed using MS Office software (Microsoft Corp., USA) and Prism 7.0 (GraphPad Software, USA) with Student's *t*-test.

## RESULTS

### Reference compounds

As a result of searching and processing information of the IUPHAR pharmacological database [5], a database containing information about the structure and experimental affinity of 48 individual reference melatonin isoster compounds was created. These structures were subjected to pharmacophore analysis and used as references in the evaluation of the molecules under investigation as described below.

### Search algorithm

The general scheme for assessing the prospectivity of compounds is depicted in Figure 2. In total, the analyzed database contained 2457 individual compounds (Fig. 3). The calculated values of physicochemical characteristics were used to evaluate drug similarity and bioavailability for topical application as eye drops (Fig. 4, 5). Substructural analysis was used to identify markers of specific toxicity (Table 1). Compounds were ranked according to the likelihood of mutagenic, carcinogenic, locally irritating properties and reproductive toxicity into three levels: no risk, low risk and high risk which was considered in the calculation of the *DrugScore* integral drug prospectivity index along with drug similarity characteristics.

Molecular pharmacophore descriptors served as descriptors of the molecules when assessing similarity to reference melatonin receptor agonists; maximum values were considered (Figs. 3, 5). Compounds that, according to at least one descriptor, had similarity to reference antagonists or inverse agonists of $MT_1$ and $MT_2$ receptors were screened out (Fig. 6).

The overall *F* prospectivity score consisted of the maximum pharmacophore similarity to the reference standards (factor weight 1.0), with consideration of *DrugScore* drug suitability index (factor weight 0.25) and the calculated corneal permeability *LogPapp* (factor weight 0.25) to account for topical application as eye drops (Table 2). The *F* indicator can take values from 0 (minimum prospectivity) to 1 (maximum prospectivity).

A visualisation of the contribution of pharmacophore affinity to melatonin analogues and calculated *LogPapp* corneal permeability to the overall *F* prospectivity score of is presented in Figure 7. The compounds with $F > 0.85$, ranked according to the comprehensive prospectivity assessment, are summarized in Table 3. They are distinguished by a set of favourable prognostic characteristics: specific types of toxicity (mutagenic, carcinogenic, locally irritating properties and reproductive toxicity) are not expected; the calculated permeability of the *LogPapp* cornea exceeds -5.43; pharmacophore similarity to the reference compounds ranges from 0.81 to 0.91, according to the Tanimoto coefficient.

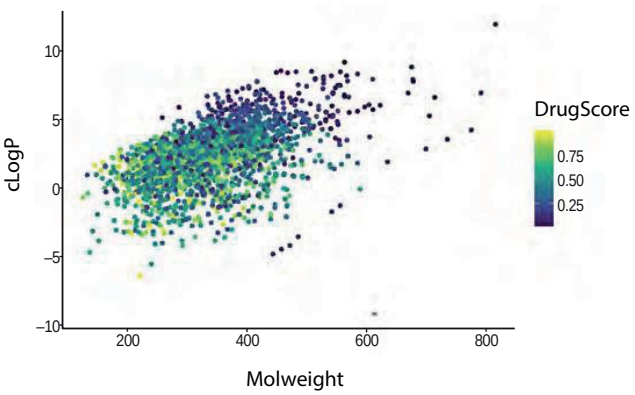



***FIG. 3.***
*Library of analyzed compounds*

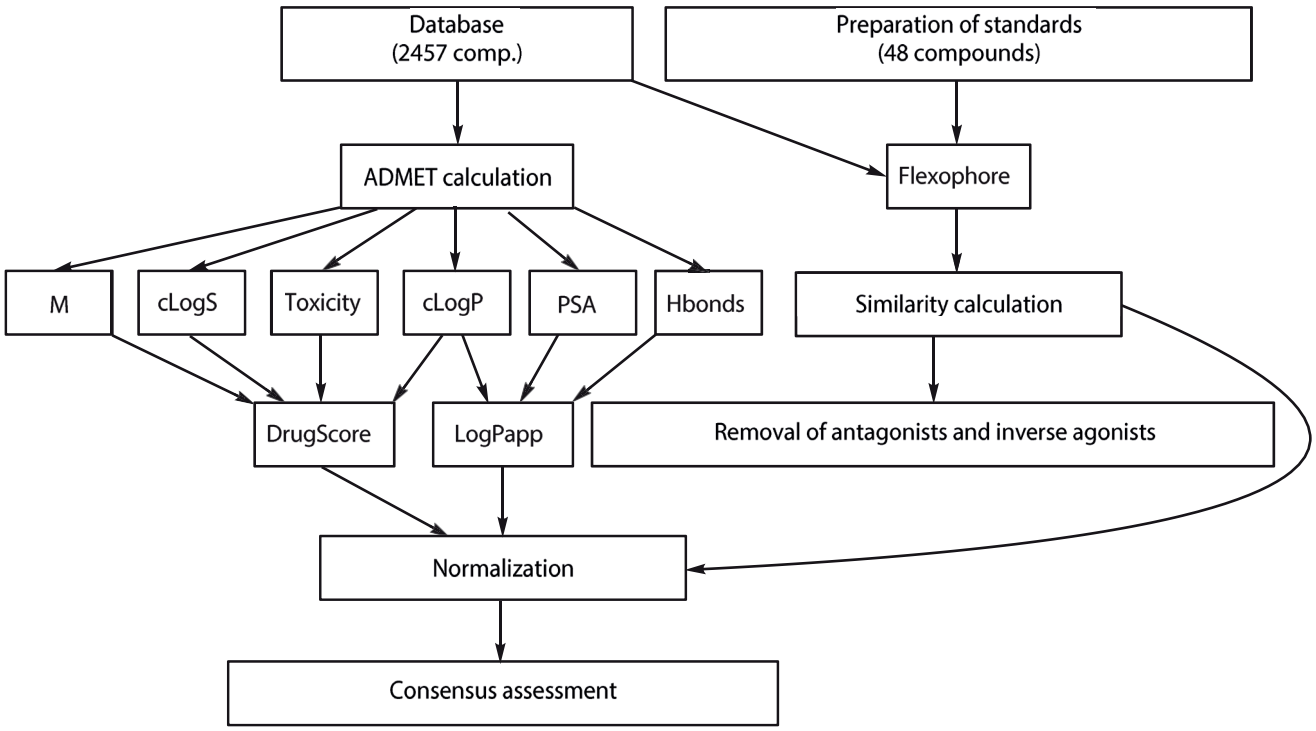



***FIG. 2.***
*Algorithm for evaluating compounds*

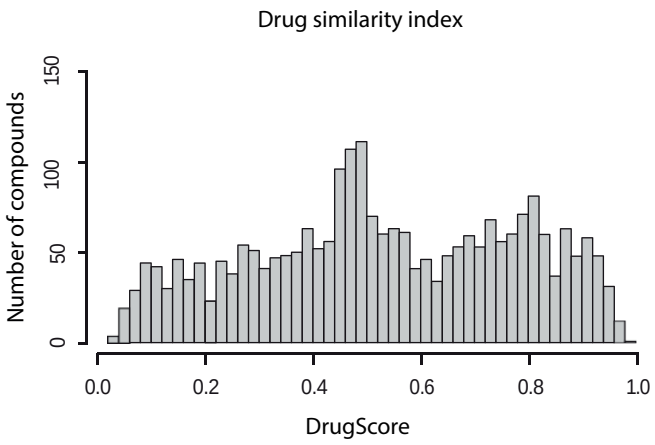



***FIG.4.***
*Drug-like distribution of compounds*

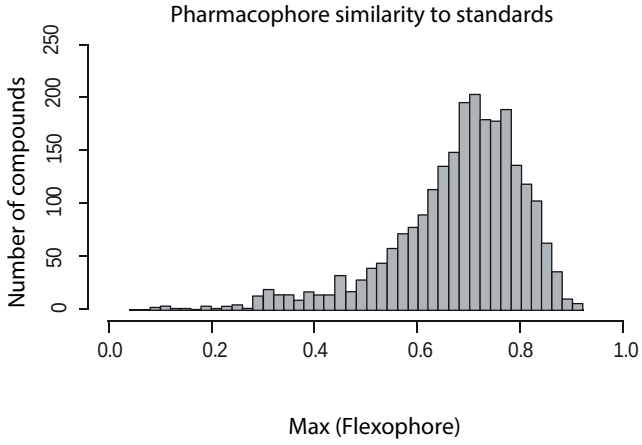



***FIG.6.***
*Distribution of compounds by pharmacophore similarity to standards*

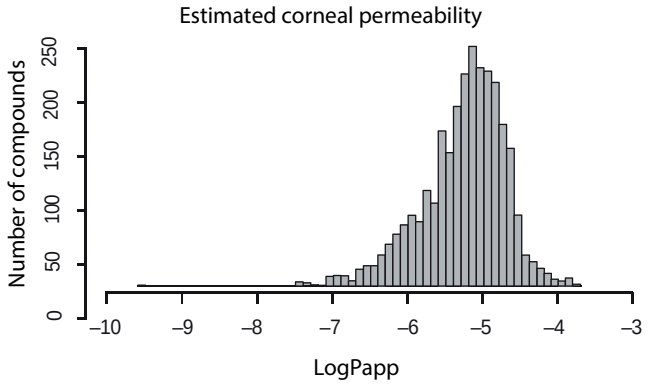



***FIG.5.***
*Distribution of compounds by calculated corneal permeability*

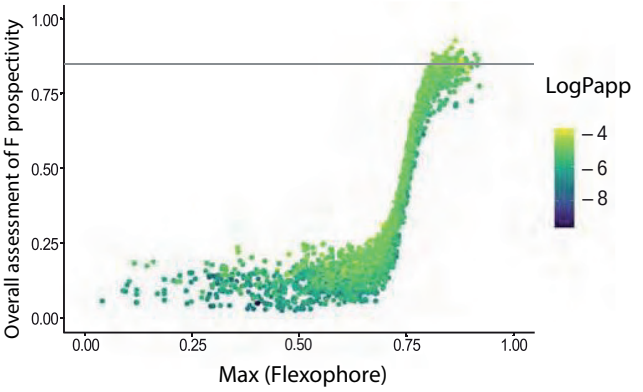



***FIG.7.***
*The contribution of pharmacophore similarity to the Max(Flexophore) standards and the calculated corneal permeability of LOGPPP to the overall F prospectivity score*

**TABLE 1**

**RESULTS OF THE SPECIFIC TYPES OF TOXICITY ASSESSMENT FOR THE STUDIED COMPOUNDS**

| Type of toxicity | Number of compounds (*n*) | | |
|---|---|---|---|
| | high risk | low risk | no risk |
| Mutagenicity | 204 | 60 | 2193 |
| Carcinogenicity | 249 | 84 | 2124 |
| Reproductive | 135 | 131 | 2191 |
| Local irritant effect | 333 | 63 | 2061 |

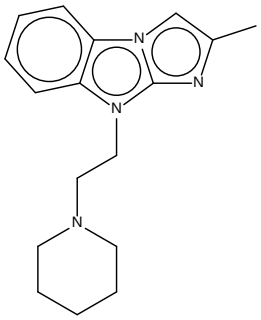
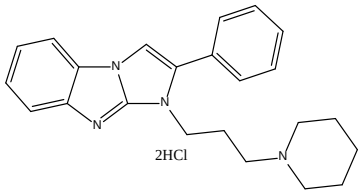
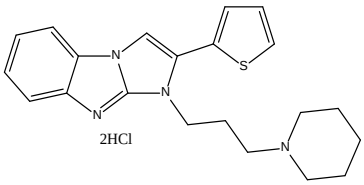
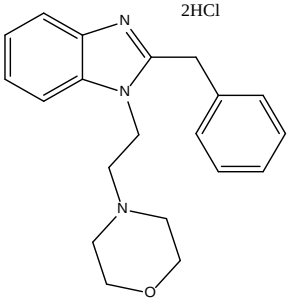
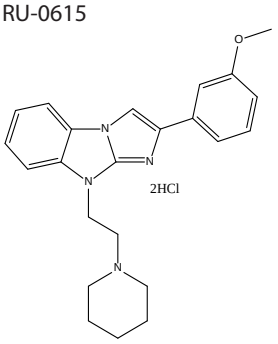
**TABLE 2**

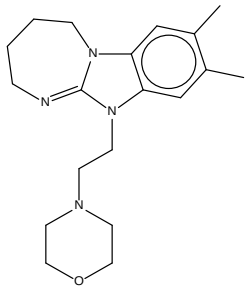
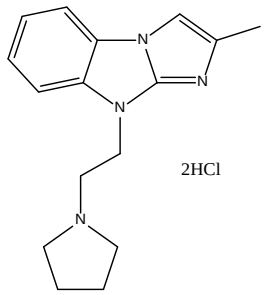
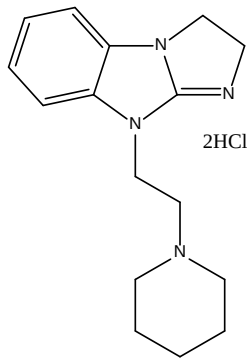
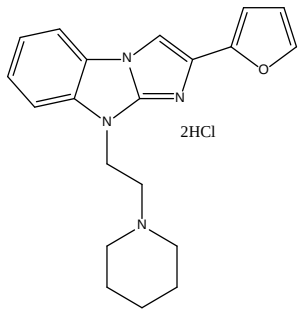
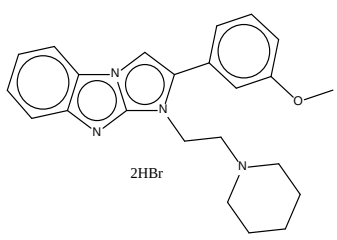
**SELECTING THE DECISIVE RULE PARAMETERS**

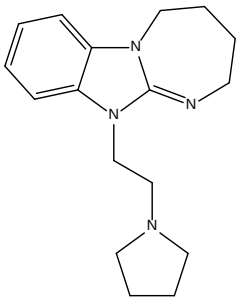
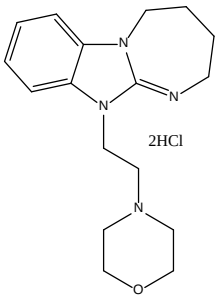
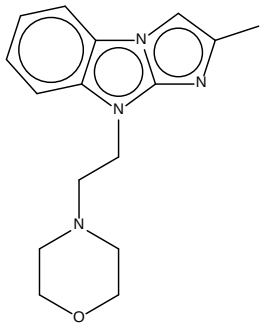
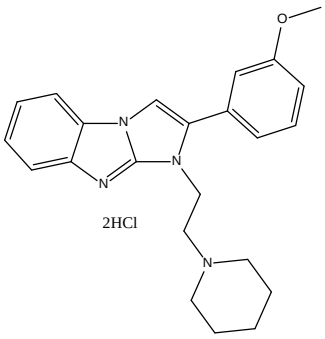
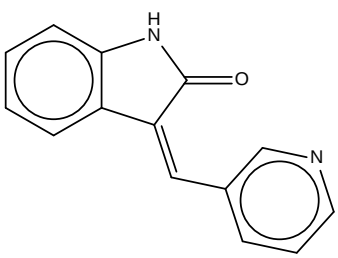
| Indicator, *p* | Condition, *a* | Slope of the curve, *b* | Weight, *k* |
|---|---|---|---|
| *DrugScore* | > 0.5 | 0.1 | 0.25 |
| *LogPapp* | ≥ 5 | 0.35 | 0.25 |
| *Max*(*Flexophore*) | > 0.75 | 1.0 | 1.0 |

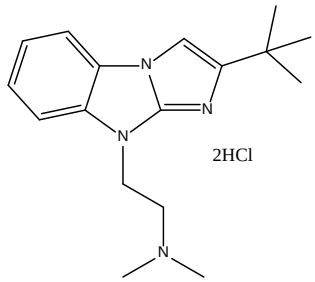
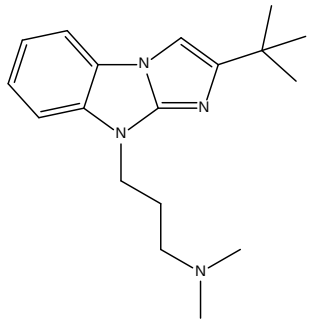
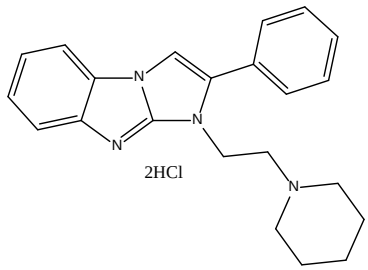
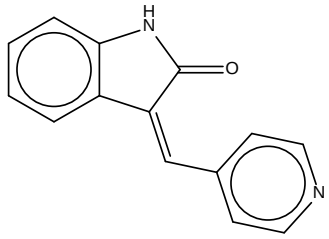
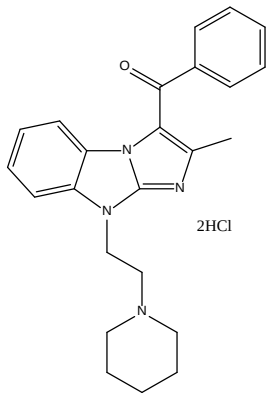
**TABLE 3**

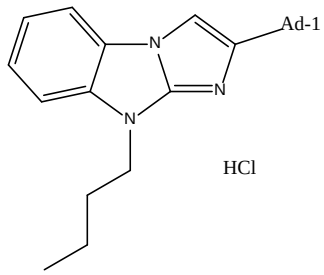
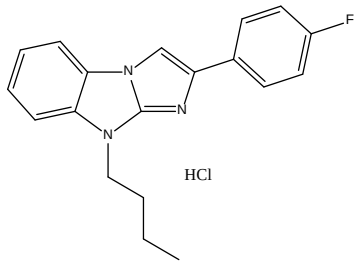
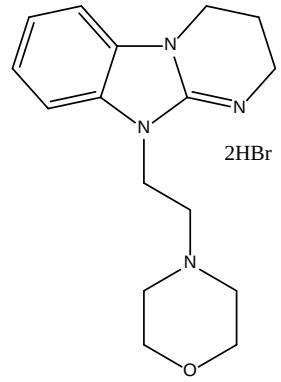
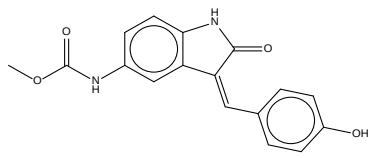
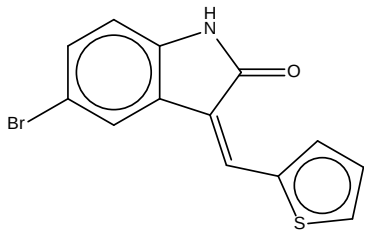
**COMPOUNDS WITH MAXIMUM PROSPECTIVITY AND THEIR IOP-LOWERING ACTIVITY**

| No. | Cipher and structure | *DrugScore*[1] | Consensus assessment *F* | IOP change, % of baseline |
|---|---|---|---|---|
| 1 | RU-1331  | 0.889 | 0.895 | –20.3 ± 3.54* |
| 2 | RU-0721  | 0.724 | 0.890 | –8.9 ± 9.62 |
| 3 | RU-0882  | 0.732 | 0.877 | –17.2 ± 14.29 |
| 4 | RU-0536  | 0.860 | 0.874 | –8.3 ± 7.54 |
| 5 | RU-0615  | 0.751 | 0.874 | –33.3 ± 4.40* |

| No. | Cipher and structure | *DrugScore*[1] | Consensus assessment *F* | IOP change, % of baseline |
|---|---|---|---|---|
| 6 | DAB-0034  | 0.799 | 0.873 | –24.5 ± 4.44* |
| 7 | RU-0026  | 0.743 | 0.872 | –11.9 ± 7.83 |
| 8 | RU-0255  | 0.865 | 0.872 | –12.4 ± 8.60 |
| 9 | RU-0155  | 0.823 | 0.871 | –2.5 ± 2.12 |
| 10 | RU-0580b  | 0.751 | 0.871 | –10.1 ± 1.70 |

| No. | Cipher and structure | *DrugScore*[1] | Consensus assessment *F* | IOP change, % of baseline |
|---|---|---|---|---|
| 11 | DAB-0021  | 0.677 | 0.870 | –17.3 ± 2.40* |
| 12 | DAB-0023  | 0.861 | 0.870 | –20.5 ± 1.43* |
| 13 | RU-1332  | 0.930 | 0.870 | –24.3 ± 4.60* |
| 14 | RU-0580  | 0.751 | 0.869 | –16.7 ± 8.33 |
| 15 | OIP-H-0003  | 0.903 | 0.866 | –5.6 |

| No. | Cipher and structure | *DrugScore*[1] | Consensus assessment *F* | IOP change, % of baseline |
|---|---|---|---|---|
| 16 | RU-0470  | 0.804 | 0.865 | –10.26 ± 10.26 |
| 17 | RU-0398  | 0.819 | 0.864 | –40.0 ± 4.15* |
| 18 | RU-0354  | 0.771 | 0.863 | –27.0 ± 5.18* |
| 19 | OIP-H-0004  | 0.903 | 0.862 | –26.11 ± 3.15* |
| 20 | RU-0514  | 0.722 | 0.862 | –2.5 ± 6.75 |

| No. | Cipher and structure | *DrugScore*[1] | Consensus assessment *F* | IOP change, % of baseline |
|---|---|---|---|---|
| 21 | RU-0012   | 0.576 | 0.861 | –6.1 ± 3.09 |
| 22 | RU-1256   | 0.541 | 0.858 | –15.71 ± 17.93 |
| 23 | RU-0837   | 0.909 | 0.856 | –5.8 ± 3.02 |
| 24 | K-00165   | 0.298 | 0.856 | –40.9 ± 3.52* |
| 25 | OIP-Br-S-1   | 0.734 | 0.851 | –13.8 ± 9.09 |

**Note.** [1] is an integral indicator that takes into account drug similarity, solubility, the risk of mutagenic, carcinogenic, irritating properties and reproductive toxicity; * – $p < 0.05$ relative to baseline IOP (*t*–test; $n = 6$).

**Validation by *in vivo* experiment**

After virtual screening of 2457 structures, 25 selected compounds with maximum *F* prospectivity score were experimentally studied to determine the effect on intraocular pressure of intact rats. 10 compounds were identified that statistically significantly reduced the IOP of intact rats. It was revealed that new bioisosters of melatonin – compounds RU-398 and K-165 – were superior to melatonin itself in their ability to reduce IOP, and compound RU-615 was not inferior to melatonin in its activity. Specifically, substance RU-398 reduced IOP by 40 %, K-165 by 40.9 %, and RU-615 and melatonin by 33.3 %. Compounds RU-398 and RU-615 also resulted in IOP reduction in the control eye, which may indicate a possible systemic action of the compounds, which was not detected for substance K-165.

## DISCUSSION

Multiparametric optimisation is one of the central and most challenging problems in the development of new drugs. To have a chance to reach the stage of clinical trials, the molecule must have a number of optimal characteristics determined by its structure - not only affinity to the biological target, but also selectivity of action, sufficient solubility, ability to penetrate tissue barriers, metabolic stability, low toxicity. The approach offered in this study is characterised by simplicity, accessibility, and flexibility. Using chemoinformatics methods, compounds with low calculated toxicity, high drug similarity and permeability through the cornea of the eye and primarily pharmacophore close to known modulators, melatonin receptor agonists, are prioritised, followed by validation by experimental screening.

The limitations of the present study include the limited sample library of source structures for the study. Additionally, experimental validation was performed by phenotypic screening for the ability to reduce intraocular pressure. Non-verification of the effect against melatonin receptors itself does not exclude the possibility of other mechanisms of action of the active compounds, different from the mechanism of action of melatonin itself.

## CONCLUSION

A flexible computational approach for prioritisation of compounds with high drug similarity, low computational toxicity and similarity to a target-oriented library of reference compounds is proposed. The effectiveness of the proposed search system was confirmed by the identification of new chemical classes and scaffolds of melatonin bioisosters promising for further study as antiglaucoma agents.

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**Conflict of interest**

The authors declare no conflict of interest.

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# CHANGES IN ACCOMMODATION DISORDERS IN CHILDREN WITH ANISOMETROPIC AMBLYOPIA AND HYPERMETROPIA

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## ABSTRACT

***Background.*** *Accommodation disorders in children with hypermetropia is one of the main factors in emmetropization disorders and maintenance of image defocusing. The most severe changes in accommodation are observed in children with anisometropia and hyperopia.*
***The aim of the work.*** *To evaluate the changes in the accommodative function of the eye in children with hyperopia, amblyopia, who underwent refractive laser surgery (RLS), as well as in children with spectacle and contact lens correction in combination with pleoptic treatment.*
***Material and methods.*** *Group 1 consisted of 30 children after RLS; group 2 consisted of 29 children who had spectacle correction; group 3 consisted of 26 children who had soft contact lens correction; all children received pleoptic treatment. Clinical examination included the analysis of objective reserves of relative accommodation (RRA) and objective accommodative response (OAR) with an open field autorefractometer, and the results of accommodation measurement.*
***Results.*** *In 1.5 years, statistically significant changes were observed in the coefficient of accommodation response (CAR) of the amblyopic eye between the groups 1 and 2 – 0.12 ± 0.02 and 0.00 ± 0.1 relative units, respectively (p = 0.01). Similar statistically significant changes were obtained in OAR and objective RRA of the amblyopic eye. At the end of the observation, the OAR in the group 1 was -2.1 ± 0.67 dpt, the objective RRA – -2.1 ± 0.67 dpt; in the group 2 the OAR was – 1.38 ± 0.19 dpt (p = 0.01), the objective RRA – -1.38 ± 0.19 dpt (p = 0.01). There were no statistically significant changes in these parameters of the amblyopic eye between the groups 1 and 3.*
***Conclusion.*** *Refractive laser and contact correction provide reduction of accommodative disorders in children with anisometropia, amblyopia and hypermetropia. After RLS there was a tendency to more close to normal CAR, OAR and objective RRA indices due to the reduction of refractive indices of the amblyopic eye.*

***Key words:*** *accommodation, amblyopia, hyperopia, anisometropia, refractive laser surgery*

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# ИЗМЕНЕНИЕ НАРУШЕНИЙ АККОМОДАЦИИ У ДЕТЕЙ С АНИЗОМЕТРОПИЧЕСКОЙ АМБЛИОПИЕЙ И ГИПЕРМЕТРОПИЕЙ

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## РЕЗЮМЕ

***Обоснование.*** *Нарушение аккомодации у детей с гиперметропией является одним из главных факторов нарушения процесса эмметропизации и поддержания расфокусировки изображения. Наиболее тяжёлые изменения аккомодации наблюдаются у детей с анизометропией и гиперметропией.*
***Цель работы****. Оценить изменения аккомодационной функции глаза у детей с гиперметропией, амблиопией, которым была выполнена рефракционная лазерная операция (РЛО), а также у детей с очковой и контактной коррекцией в сочетании с плеоптическим лечением.*
***Материал и методы.*** *В 1-ю группу вошли 30 детей после РЛО, во 2-ю группу –29 детей с очковой коррекцией, в 3-ю группу – 26 детей с контактной коррекцией; все дети получали плеоптическое лечение. Клиническое исследование включало анализ объективных запасов относительной аккомодации (ЗОА) и объективного аккомодационного ответа (ОАО) на авторефрактометре открытого поля, результатов аккомодограммы на аккомодографе.*
***Результаты.*** *Через 1,5 года отмечались статистически значимые изменения коэффициента аккомодационного ответа (КАО) амблиопичного глаза между 1-й и 2-й группами, где он составил 0,12 ± 0,02 и 0,00 ± 0,1 усл. ед. соответственно (p = 0,01). Аналогичные статистически значимые изменения были получены среди ОАО и объективных ЗОА амблиопичного глаза. В конце наблюдения ОАО 1-й группы составил –2,1 ± 0,67 дптр, объективные ЗОА – –2,1 ± 0,67 дптр, во 2-й группе ОАО составил –1,38 ± 0,19 дптр (p = 0,01), объективные ЗОА – – 1,38 ± 0,19 дптр (p = 0,01). Статистически значимых изменений данных показателей амблиопичного глаза между 1-й и 3-й группами зарегистрировано не было.*
***Заключение.*** *Рефракционная лазерная и контактная коррекция обеспечивают снижение аккомодационных нарушений у детей с анизометропией, амблиопией и гиперметропией. После РЛО отмечена тенденция к более близким к норме показателям КАО, ОАО и объективных ЗОА за счёт снижения рефракционных показателей амблиопичного глаза.*

***Ключевые слова:*** *аккомодация, амблиопия, гиперметропия, анизометропия, рефракционная лазерная хирургия*

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## BACKGROUND

Refractive anomalies are the primary cause of reduced visual acuity [1]. The prevalence of hypermetropia is influenced by a wide range of factors including ethnicity and socio-economic development [2]. Approaches to laser correction of hypermetropia require a comprehensive evaluation [3]. Hypermetropia correction tactics in children among doctors are ambiguous and include prescribing full and partial inadequate correction. In children with hypermetropia, correction is usually incomplete. Initially, the accommodation of children must overcome small hyperopic errors [4]. As a second, since a fuzzy image stimulates increased accommodation work and thus promotes emmetropisation, full correction may prevent this by removing the tendency of blur to emmetropise [5]. These assumptions have recently been questioned due to lack of objective evidence [6]. Children with moderate to severe hyperopia and amblyopia are not equipped to cope with residual hyperopia and thus are unlikely to be equipped to cope with hyperopia remaining after inadequate correction [7].

Accommodation disorders in children with hypermetropia is one of the main factors in emmetropization disorders and maintenance of image defocusing. The most severe changes in accommodation are observed in children with anisometropic amblyopia and hypermetropia, who develop anisoaccommodation and weakness of accommodation [8]. Contact correction is preferred in these children to avoid persistent functional impairment and to ensure full correction. In contrast to glasses, contact lenses more accurately convey the size of objects and the distance between them, contributing to the formation of a more correct worldview in the child [9]. It should be emphasised that spectacle and contact correction for anisometropia greater than 3 dpt cannot always ensure full rehabilitation of patients both clinically and socially. Therefore, refractive laser surgery (RLS) is one of the promising methods of correction of complex ametropia, which, according to the analysed literature, has proven to be safe and effective.

The issue of studying accommodative changes in hypermetropia, especially in anisometropia, remains incompletely understood. The prevention and rehabilitation of various types of accommodation disorders in children with hyperopic amblyopia and anisometropia also remain poorly studied.

## THE AIM

To evaluate changes in the accommodative function of the eye in children with hypermetropia, amblyopia who underwent refractive laser surgery, as well as in children with spectacle and contact correction combined with pleoptic treatment.

## MATERIAL AND METHODS

A total of 86 children aged 6 to 15 years with anisometropic amblyopia and hypermetropia were treated and divided into three groups. Group 1 included 30 children after RLS, Group 2 included 29 children with spectacle correction, and Group 3 included 26 children with contact correction. Inclusion criteria for the study were the presence of hypermetropia of 3.0 dpt or more, anisometropia of more than 3 dpt, and visual acuity of the amblyopic eye of 0.05 to 0.3. Children with strabismus were excluded from the sample. No statistically significant changes were revealed between the groups in terms of sex and age. Before and after treatment every 6 months within 1.5 years, all children underwent ophthalmological examination consisting of visometry, determination of retinal visual acuity, autorefractometry, biomicroscopy and ophthalmoscopy; additionally, the state of accommodation was examined in all children: objective relative accommodation reserve (RRA) and objective accommodative response (OAR) were studied on an open-field autorefractometer WR-5100K (Grand Seiko, Japan); accommodation was analysed using Righton Speedy-K (USA).

The OAR study was performed in a contact lens providing full correction of hyperopia, with binocular fixation of the gaze on the target at a distance of 33 cm and recording the results of each eye separately. Objective RRAs were determined by adding negative lenses with a power of -0.5 until no data were recorded close to the OAR.

During the study of accommodation on the Righton Speedy-K accommodationograph (USA), changes in the eye refraction were recorded in the form of a bar chart when a visual stimulus was presented at different distances with fixation of the value of accommodation response coefficients and microfluctuations.

The ethical review of the study was conducted at a meeting of the Local Ethical Committee of the S.N. Fedorov Eye Microsurgery Research Centre of the Ministry of Health of Russia (April 1, 2021, Minutes № 104.7).

Statistical processing of data was performed using Statistica 10 (StatSoft Inc., USA) and MS Excel 2007 (Microsoft Corp., USA) software. Statistically significant differences were determined using the criteria of nonparametric and parametric statistics: the Kruskall – Wallis test was used for independent samples, and the Wilcoxon test was used for dependent samples. A $p$ value of $< 0.05$ was considered as a condition for determining statistically significant differences.

The uncorrected visual acuity (UCVA) of the amblyopic eye before treatment was 0.09 ± 0.06 in group 1, 0.11 ± 0.07 ($p = 0.24$) in group 2, and 0.13 ± 0.06 ($p = 0.16$) in group 3. Corrected visual acuity (CVA) of the amblyopic eye was 0.12 ± 0.1 in preoperative children, 0.14 ± 0.09 ($p = 0.53$) in spectacle-corrected children, and 0.15 ± 0.08 ($p = 0.49$) in contact-corrected children. Before treatment, the spherical equivalent (SE) of the amblyopic eye was +6.77 ± 1.8 dpt in group 1, +7.13 ± 3.3 dpt in group 2 ($p = 0.21$), and +5.91 ± 2.8 dpt in group 3 ($p = 0.26$). The degree of anisometropia in SE was +4.25 ± 1.4 dpt, +5.7 ± 1.9 dpt ($p = 0.09$) and +4.9 ± 2.4 dpt ($p = 0.32$) in groups 1, 2 and 3, respectively. No statistically significant difference between the refractive indices of the patients

was observed during the analysis using the Kruskall – Wallis criterion, indicating the homogeneity of the three groups. The UCVA and CVA of the paired eye were close to 1.0, and refraction was represented by emmetropia or mild hypermetropia.

## RESULTS AND DISCUSSION

Statistically significant changes were revealed after 1.5 years against the background of treatment methods when comparing the three groups. The UCVA of the amblyopic eye was 0.25 ± 0.07 in group 1, 0.12 ± 0.08 in group 2 ($p$ = 0.05), and 0.15 ± 0.05 in group 3 ($p$ = 0.01). The CVA of the amblyopic eye in Group 1 in children after refractive surgery combined with pleoptic treatment reached 0.39 ± 0.04 by the end of the observed period, in the group of children with spectacle correction and pleoptic treatment the ROC was the lowest -0.19 ± 0.08 ($p$ = 0.00), in the group of children with contact correction combined with pleoptic treatment the ROC was 0.31 ± 0.1 ($p$ = 0.06). In the group of children after refractive surgery, the SE

**TABLE 1**

**ACCOMODOGRAM COEFFICIENTS OF THE AMBLYOPIC EYE IN CHILDREN WITH HYPEROPIC ANISOMETROPIA AND AMBLYOPIA IN THREE COMPARISON GROUPS ($n$ = 86)**

| Indicators | Standard | Groups | Before treatment | 6 m | 1 year | 1.5 years | $p_w$ |
|---|---|---|---|---|---|---|---|
| CAR, c.u. | 0.25–0.65 | 1st ($n$ = 30) | 0.01 ± 0.08 | 0.06 ± 0.08 | 0.1 ± 0.06 | 0.12 ± 0.02 | **0.02** |
| | | 2nd ($n$ = 29) | –0.03 ± 0.15 | –0.03 ± 0.1 | –0.02 ± 0.14 | 0.00 ± 0.1 | 0.1 |
| | | *p value* | 0.09 | 0.07 | 0.15 | **0.01** | |
| | | 3rd ($n$ = 26) | 0.05 ± 0.08 | 0.04 ± 0.12 | 0.07 ± 0.1 | 0.08 ± 0.05 | **0.04** |
| | | *p value* | 0.35 | 0.24 | 0.1 | 0.06 | |
| SC, c.u. | 0.00–0.30 | 1st ($n$ = 30) | 0.24 ± 0.09 | 0.22 ± 0.06 | 0.27 ± 0.08 | 0.23 ± 0.04 | 0.16 |
| | | 2nd ($n$ = 29) | 0.23 ± 0.12 | 0.25 ± 0.09 | 0.25 ± 0.1 | 0.26 ± 0.12 | 0.14 |
| | | *p value* | 0.34 | 0.28 | 0.19 | 0.3 | |
| | | 3rd ($n$ = 26) | 0.27 ± 0.08 | 0.24 ± 0.13 | 0.25 ± 0.12 | 0.27 ± 0.15 | 0.31 |
| | | *p value* | 0.41 | 0.25 | 0.28 | 0.11 | |
| GC, c.u. | 0.60–0.90 | 1st ($n$ = 30) | 0.49 ± 0.06 | 0.5 ± 0.06 | 0.53 ± 0.05 | 0.5 ± 0.02 | 0.13 |
| | | 2nd ($n$ = 29) | 0.47 ± 0.1 | 0.5 ± 0.09 | 0.49 ± 0.07 | 0.5 ± 0.03 | 0.11 |
| | | *p value* | 0.16 | 0.49 | 0.08 | 0.23 | |
| | | 3rd ($n$ = 26) | 0.51 ± 0.09 | 0.5 ± 0.12 | 0.5 ± 0.14 | 0.52 ± 0.09 | 0.52 |
| | | *p value* | 0.06 | 0.24 | 0.17 | 0.18 | |
| MF, μf/min | up to 57 | 1st ($n$ = 30) | 56.5 ± 4.2 | 65.6 ± 4.1 | 63.5 ± 4.1 | 64.3 ± 2.5 | **0.01** |
| | | 2nd ($n$ = 29) | 55.1 ± 5.1 | 57.8 ± 3.9 | 57.4 ± 4.0 | 59.1 ± 3.5 | **0.05** |
| | | *p value* | 0.1 | **0.01** | **0.02** | **0.03** | |
| | | 3rd ($n$ = 26) | 57.4 ± 5.5 | 59.8 ± 5.0 | 60.6 ± 4.0 | 61.5 ± 5.7 | **0.03** |
| | | *p value* | 0.12 | **0.01** | **0.04** | 0.08 | |

**Note.** Here and in Table 2: $p$ – Kruskall – Wallis test between groups; $P_w$ – Wilcoxon intra-group criterion; SC – stability coefficient; GC – growth coefficient; MF – microfluctuation factor.

**TABLE 2**

**ACCOMODOGRAM COEFFICIENTS OF THE PAIRED LEADING EYE IN CHILDREN WITH HYPEROPIC ANISOMETROPIA AND AMBLYOPIA IN THREE COMPARISON GROUPS (*n* = 86)**

| Indicators | Standard | Groups | Before treatment | 6 m | 1 year | 1.5 years | $p_w$ |
|---|---|---|---|---|---|---|---|
| CAR, c.u. | 0.25–0.65 | 1st (*n* = 30) | 0.39 ± 0.11 | 0.35 ± 0.04 | 0.41 ± 0.08 | 0.43 ± 0.1 | 0.06 |
| | | 2nd (*n* = 29) | 0.35 ± 0.24 | 0.39 ± 0.15 | 0.33 ± 0.14 | 0.34 ± 0.11 | 0.15 |
| | | 3rd (*n* = 26) | 0.41 ± 0.16 | 0.39 ± 0.19 | 0.42 ± 0.14 | 0.45 ± 0.16 | 0.07 |
| | | *p value* | 0.21 | 0.18 | 0.26 | 0.12 | |
| SC, c.u. | 0.00–0.30 | 1st (*n* = 30) | 0.28 ± 0.09 | 0.25 ± 0.07 | 0.26 ± 0.13 | 0.25 ± 0.07 | 0.24 |
| | | 2nd (*n* = 29) | 0.30 ± 0.07 | 0.28 ± 0.1 | 0.27 ± 0.15 | 0.27 ± 0.13 | 0.14 |
| | | 3rd (*n* = 26) | 0.27 ± 0.16 | 0.30 ± 0.12 | 0.28 ± 0.11 | 0.28 ± 0.14 | 0.36 |
| | | *p value* | 0.38 | 0.06 | 0.09 | 0.11 | |
| GC, c.u. | 0.60–0.90 | 1st (*n* = 30) | 0.51 ± 0.15 | 0.5 ± 0.08 | 0.52 ± 0.08 | 0.5 ± 0.13 | 0.47 |
| | | 2nd (*n* = 29) | 0.48 ± 0.08 | 0.49 ± 0.1 | 0.51 ± 0.08 | 0.52 ± 0.07 | 0.06 |
| | | 3rd (*n* = 26) | 0.52 ± 0.09 | 0.53 ± 0.12 | 0.53 ± 0.14 | 0.53 ± 0.16 | 0.41 |
| | | *p value* | 0.16 | 0.22 | 0.25 | 0.09 | |
| MF, μf/min | up to 57 | 1st (*n* = 30) | 64.8 ± 5.2 | 64.1 ± 2.7 | 62.8 ± 4.9 | 60.5 ± 2.4 | **0.05** |
| | | 2nd (*n* = 29) | 65.4 ± 4.1 | 65.1 ± 4.9 | 64.4 ± 3.8 | 64.2 ± 5.5 | 0.07 |
| | | 3rd (*n* = 26) | 0.24 | 0.26 | 0.06 | **0.04** | |
| | | *p value* | 63.2 ± 4.7 | 62.9 ± 3.6 | 62.5 ± 4.2 | 62.1 ± 4.4 | 0.06 |
| | | 1st (*n* = 30) | 0.12 | **0.05** | 0.18 | 0.06 | |

of the amblyopic eye was +1.23 ± 0.11 dpt, the degree of anisometropia according to SE was +1.25 ± 1.4 dpt; in Groups 2 and 3, these values were at baseline; the difference between the groups was statistically significant ($p < 0.001$). Consequently, all three groups observed an increase in visual acuity on the background of the treatment.

The data of the accommodation coefficients of the amblyopic eye are summarised in Table 1, of the leading paired eye in Table 2.

The accomodogram of the amblyopic eye had a gentle course due to a low response to the visual stimulus; «dips» were also recorded, which are the result of the lack of response of the ciliary muscle to the visual stimulus. In the paired leading eye, habitually excessive accommodation tension was registered, manifested by a high number of microfluctuations. Anisoaccommodation was revealed in all children. According to the data of the analysis, in the amblyopic eye there was a statistically significant increase in CAR among patients after refractive laser surgery and in children with contact correction, and in all three comparison groups there was a statistically significant increase in the microfluctuation factor (MF). In the paired leading eye, a statistically significant decrease in MF in children after refractive-laser surgery was found, which is probably related to the increase in corrected visual acuity in the paired amblyopic eye and redistribution of visual load.

According to computerised accommodationography, children in the amblyopic eye showed weakness of accommodation and combined accommodation disorders manifested as low accommodation response coefficient and high microfluctuation coefficient. S.V. Balalin and L.P. Trufanova were among the first to additionally identify this combined type of accommodation disorder [10].

E.G. Solodkova et al. (2019) used an accommodationograph to study changes in accommodation characteristic of children with hypermetropia [11]. According to their study, patients with moderate to severe hyperopia were diagnosed with accommodation weakness with low CAR

and normal MF, and a combination of accommodation weakness with habitual excessive accommodation tension – low CAR with high MF, which is also consistent with our data.

According to the open-field autorefractometer study, the OAR of the amblyopic eye was reduced in all three groups. At the beginning of follow-up, the findings were homogeneous between the groups. In the group of children after RLS the mean value of OAR was -1.1 ± 0.8 dpt, in children with spectacle correction this index was -1.22 ± 0.55 dpt ($p$ = 0.34), in children with contact correction – -1.25 ± 0.23 dpt ($p$ = 0.21). According to the Donders` formula, the norm of accommodative response was -3.0 dpt [12]. After 1.5 years of follow-up, the OAR of the amblyopic eye of group 1 was significantly close to normal and was -2.1 ± 0.67 dpt. The OAR of the 1st group was statistically significantly different from that of the 2nd group, where the OAR of the amblyopic eye was -1.38 ± 0.19 dpt ($p$ = 0.01), in the 3rd group this index reached -1.8 ± 0.4 dpt ($p$ = 0.07). In the paired leading eye, there was a decrease in OAR, but less pronounced compared to the amblyopic eye. Changes in OAR between groups in the paired leading eye as well as in the amblyopic eye were not statistically significant at the beginning of observation. The OAR of the paired leading eye in groups 1, 2 and 3 were -1.9 ± 0.4 dpt, -1.8 ± 0.56 dpt ($p$ = 0.69) and -1.9 ± 0.42 dpt ($p$ = 0.86), respectively. After 1.5 years of treatment, OAR also increased in the paired lead eye; no statistically significant differences between groups were found. In children after RLS this index was -2.4 ± 0.24 dpt, after spectacle correction – -2.2 ± 0.13 dpt ($p$ = 0.11), after contact correction – -2.4 ± 0.24 dpt ($p$ = 0.32).

The values of objective RRA of the amblyopic eye were reduced: in children after RLS and with spectacle contact correction the initial data of objective RRA were close and were -1.0 ± 0.2 and -1.0 ± 0.3 dpt ($p$ = 0.89), respectively; in children with contact correction this index was at -1.2 ± 0.82 dpt ($p$ = 0.54), respectively. Age norms of relative accommodation reserve were determined by E.S. Avetisov and K.M. Matz (1971) as -3.0 ÷ -5.0 dpt [13]. After treatment, objective RRA in children after RLS was -2.1 ± 0.67 dpt, in children with spectacle correction – -1.38 ± 0.19 dpt ($p$ = 0.01), with contact correction – -1.8 ± 0.4 dpt ($p$ = 0.06). Thus, all groups showed an increase in objective RRA of the amblyopic eye; statistically significant changes were observed only between groups 1 and 2. The registration of reduced indices of objective RRA was also revealed in the paired leading eye, which is probably related to the presence of concomitant accommodation. Initial objective RRA data of the paired eye in the group of children after RLS were -2.0 ± 0.48 dpt, in children with spectacle correction -1.8 ± 0.32 dpt ($p$ = 0.34), and in the group of children with contact correction -2.0 ± 0.52 dpt ($p$ = 0.67). After 1.5 years, these values between groups remained approximate and were -2.51 ± 0.31 dpt, -2.0 ± 0.6 dpt ($p$ = 0.09), and -2.38 ± 0.42 dpt ($p$ = 0.12) in groups 1, 2, and 3, respectively.

According to the works of E.P. Tarutta et al. (2012), in hypermetropia and in myopia there is a delay in accommodative response, which increases depending on the degree of ametropia, but in myopia the delay of OAR is stronger than in hypermetropia, and it should be noted that the studies of this group of authors were conducted on children without amblyopia [14, 15].

Therefore, according to the data of the present study, the recovery of accommodation disorders in children after refractive laser surgery and with contact correction were comparable. In the above groups, there was a statistically significant increase in CAR, MF, OAR and objective RRA of the amblyopic eye compared to the group of children with spectacle correction.

## CONCLUSION

Refractive laser surgery and contact correction provide reduction of accommodation disorders in children with anisometropic amblyopia and hypermetropia. After refractive laser surgery, there was a tendency to closer to normal values of the accommodation response coefficient, objective accommodative response and objective relative accommodation reserves due to the reduction of refractive indices of the amblyopic eye

**Conflict of interest**

The authors declare no conflict of interest.

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# INTRAOCULAR DIROFILARIASIS: SURGICAL APPROACHES, FEATURES OF THE CLINICAL COURSE (CLINICAL CASE)

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## ABSTRACT

***The aim.*** *To present a clinical case of surgical treatment of parasitic invasion of the vitreous body caused by dirofilaria.*
***Material and methods.*** *One patient with intraocular dirofilariasis underwent surgical treatment including vitrectomy, phacoemulsification with implantation of an intraocular lens. At the stage of vitrectomy, a whole helminth was removed from the vitreal cavity using collet tweezers for subsequent typing. The uncorrected visual acuity of the right eye at the time of treatment was 0.4, of the left eye – 0.45.*
***Results.*** *The postoperative period had no signs of an active inflammatory reaction. Four months after surgical treatment, at a follow-up visit, visual acuity of the left eye reached 1.0. According to the parasitological study, a female Dirofilaria repens was identified. In the postoperative period, the areas of pronounced chorioretinal atrophy in the peripheral parts of the retina were visualized, which may be a consequence of mechanical contact of the parasite or the toxic effects of its metabolic products.*
***Conclusion.*** *This clinical case demonstrates the possibility of infection with the ocular form of dirofilariasis in a region that is atypical for the presence of this helminth. Despite the positive outcome of the disease, in the presented patient, the long-term presence of the parasite in the vitreal cavity led to the formation of chorioretinal atrophy in the peripheral retina, which confirms the need for timely diagnosis and surgical treatment.*

***Key words:*** *dirofilariasis, vitrectomy, zoonosis, ophthalmohelminthiasis*

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# ИНТРАОКУЛЯРНЫЙ ДИРОФИЛЯРИОЗ: ХИРУРГИЧЕСКИЕ ПОДХОДЫ, ОСОБЕННОСТИ КЛИНИЧЕСКОГО ТЕЧЕНИЯ (КЛИНИЧЕСКИЙ СЛУЧАЙ)

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## РЕЗЮМЕ

***Цель.*** *Представить клинический случай хирургического лечения паразитарной инвазии стекловидного тела, вызванной дирофиларией.*
***Материал и методы.*** *Прооперирована 1 пациентка с интраокулярным дирофилариозом. Пациентке проведено оперативное лечение в объёме витрэктомии, факоэмульсификации катаракты с имплантацией интраокулярной линзы. На этапе проведения витрэктомии цанговыми пинцетами произведено удаление цельного гельминта из витреальной полости для последующего типирования. Некорригированная острота зрения правого глаза на момент обращения составляла 0,4, левого – 0,45.*
***Результаты.*** *Послеоперационный период протекал без признаков активной воспалительной реакции. Через 4 месяца после хирургического лечения, на контрольной явке, острота зрения левого глаза достигла 1,0. По данным паразитологического исследования идентифицирована самка Dirofilaria repens. В послеоперационном периоде у пациентки визуализировались зоны выраженной хориоретинальной атрофии периферических отделов сетчатки, что, возможно, является последствием механического контакта паразита либо токсического воздействия продуктов его жизнедеятельности.*
***Заключение.*** *Данный клинический пример демонстрирует возможности заражения глазной формой дирофилариоза в нетипичном для нахождения данного гельминта регионе. Несмотря на положительный исход заболевания, у представленной пациентки длительное нахождение паразита в витреальной полости привело к формированию хориоретинальной атрофии на периферии сетчатки, что подтверждает необходимость проведения своевременной диагностики и оперативного вмешательства.*

***Ключевые слова:*** *дирофилариоз, витрэктомия, зооноз, отфальмогельминтоз*

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Dirofilariasis (from Latin «*diro*, *filum*» – «evil thread») is a disease caused by parasitisation of nematodes of the genus *Dirofilaria* in the human body [1]. Dirofilariae are thin, white-coloured helminths belonging to the class of roundworms-nematodes. About twenty species of Dirofilariae are known, but *Dirofilaria repens* and *Dirofilaria limmitis* are particularly dangerous for humans. The female *Dirofilaria repens* reaches a length of 130–150 mm, *Dirofilaria limmitis* reaches a length of 180–300 mm, while male *Dirofilaria repens* reaches a length of 50–60 mm, male *Dirofilaria limmitis* reaches a length of 100–110 mm. Generally, the helminth can be 0.03 to 1.2 mm wide [2, 3]. *Dirofilaria limmitis* causes the pulmonary form of the disease, *Dirofilaria repens* the oculocutaneous form. An infected mosquito can infect humans with dirofilariasis from the genera *Culex*, *Aedes*, and *Anopheles* [1, 4, 5]. The mosquito is not the only vector of the disease; there have been known cases of invasion following bites from mites, horseflies, lice and fleas. Until recently, it was thought that the larva grows in tissues of the human body but does not undergo sexually mature changes. But the literature describes cases of microfilaraemia, which suggests the possibility of infection of the organism by at least two individuals of different sexes with their transition to the sexually mature stage of development and that a person can be the definitive carrier of *Dirofilaria repens* [5]. In 1566, the Portuguese physician Lusitano Amato (1511–1568) first described a case of a worm being removed from the eye of a little girl. In 1915, the Russian physician A.P. Vladychensky described a case of extracting a worm from a patient's eye tumour [6, 7, 8]. A significant contribution to the study of dirofilaria was made by the Soviet helminthologist K.I. Skryabin in 1917–1948. Ocular dirofilariasis with involvement of the visual organ accounts for 38 to 88 % of cases [8]. In 1996, T.I. Avdyukhina et al. compiled a registry of patients with dirofilariasis, in which 50 out of 110 cases accounted for dirofilariasis of the visual organ [9]. Intraocular localisation is considered a rare form of ocular dirofilariasis. For example, 5 cases of ocular dirofilariasis with intraocular localisation have been registered in the Russian Federation over the years [8].

Currently, the increasing incidence of dirofilariosis is again attracting the attention of physicians [10]. The main reasons for the increase in morbidity and expansion of the area of dirofilariasis spread, according to scientists, are changes in climatic, social and environmental conditions, as well as the growth of migration activity of the population [11]. Until recently, the disease was thought to be predominantly prevalent in Asia, Africa and Southern Europe, but in recent years there has been an increase in the number of cases of dirofilariasis in Nordic countries (Finland, Estonia), where the disease was previously extremely rare [12]. In the Russian Federation, cases of dirofilariasis were previously registered mainly in the southern regions, but now there are cases in St. Petersburg, Tomsk, Irkutsk, and Sverdlovsk regions [13]. Given the polymorphism of clinical manifestations of ocular dirofilariasis, diagnosis of the disease is complicated, and often the final diagnosis is made during surgical treatment with extraction of the parasite and its subsequent typing. Thus, dirofilariasis is a complex and interdisciplinary disease that requires increased attention of physicians at the place of residence, especially in regions not endemic for this pathology.

## THE AIM

To present a clinical case of surgical treatment of parasitic invasion of the vitreous body caused by dirofilaria.

## MATERIALS AND METHODS

Patient T., 59 years old, came to the ophthalmological clinic at her place of residence with complaints of reduced vision in the left eye for several years and the presence of a floating formation in front of it in the form of a «thread» that had been bothering her for six months. Ophthalmological examination revealed a mobile formation in the lower segment of the vitreal cavity. The diagnosis was made: parasitic invasion of the vitreous body; the patient was referred for surgical treatment. A complete ophthalmological examination including visometry, intraocular pressure (IOP) measurement, autorefractometry, anterior eye segment biomicroscopy, perimetry, ophthalmoscopy of the ocular fundus and B-scan ultrasonography was performed at the time of presentation for surgery. The incorrigible (i.c.) visual acuity of the right eye (OD, oculus dexter) at the time of treatment was 0.4, of the left eye (OS, oculus sinister) – 0.45, IOP (p) of both eyes – 11 mmHg. Significant opacities in the cortex of the crystalline lens were revealed, during the examination of the eye fundus a floating conglomerate of the vitreous body with a translucent mobile formation of thread-like shape was diagnosed. Areas of chorioretinal atrophy were also revealed in the middle and extreme periphery, while the optic disc and macula remained within normal limits. Ultrasound B-scan confirmed the presence of a large focus of vitreous opacity, within which a mobile object was observed (Fig. 1). Diagnosis: vitreous opacity, parasitic invasion of the vitreous, incomplete complicated cataract of the left eye.

A clinical decision was made to perform combined treatment – posterior closed vitrectomy and cataract phacoemulsification with intraocular lens (IOL) implantation. In order to minimise the risks of inflammation when the parasite is destroyed in response to foreign antigens, and in order to typify the helminth, surgical approaches were used to remove the parasite without damaging it. Surgical treatment was performed using Alcon Constellation system (Alcon, USA). The first stage was cataract phacoemulsification without IOL implantation. Three 25-gauge ports were installed using standard technique, 3 mm from the limb. An infusion cannula was placed into the port in the lower outer segment. A 4 mm diameter posterior capsulorhexis was formed with a vitrectomy cutter to extract the parasite through it. When capsulorhexis was performed, the vitreous conglomerate with the parasite

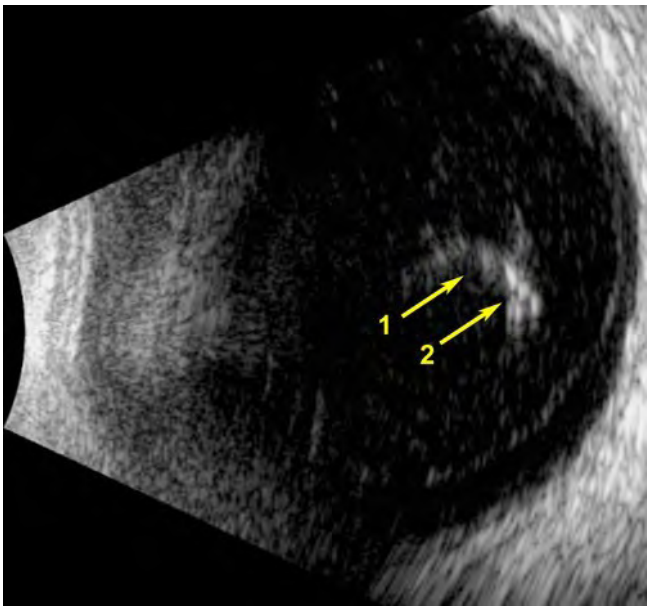


***FIG. 1.***
*B-scan. Vitreous body and parasite conglomerate: 1 - vitreous opacity; 2 - mobile hyperechogenic formation*

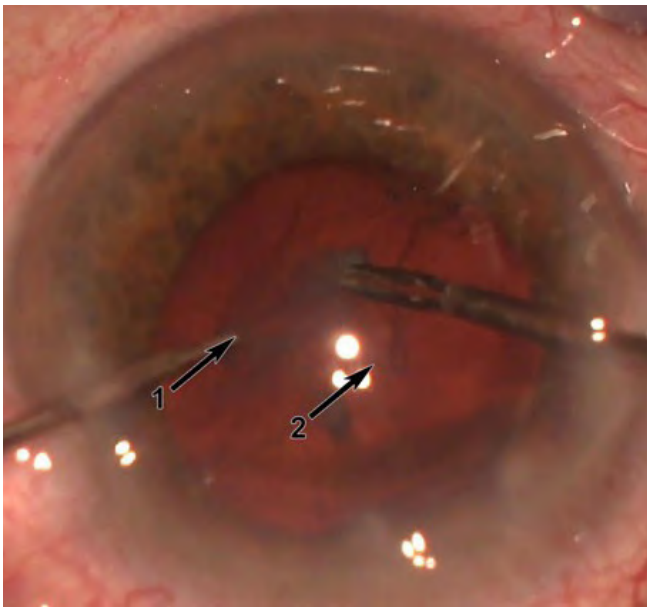


***FIG. 2.***
*Photoregistration of the stage of surgical intervention: 1 – vitreous opacity; 2 – parasite embedded in the fibres of the vitreous body*

migrated into the retrolenticular space. Through paracentesis, the conglomerate was fixed with 25G collet forceps and an attempt was made to remove the helminth with forceps from the vitreous body (Fig. 2). The attempt was unsuccessful, however, because of the pronounced fixation of the parasite in the fibres of the vitreous body. A decision has been made to change the helminth extraction technique. The fibres of the vitreous body fixed with conglomerate were resected with a vitrectomy cutter. At the time of vitrectomy, there was marked mobility of the parasite with a tendency to migrate to the vitrectomy cutter window and risk of damage.

It should be emphasised that intraoperatively the possibility of administration of 0.01 % carbachol solution was considered in order to reduce helminth mobility, but possible miosis and risks of reduced visualisation led to the rejection of this drug. With forceps, the conglomerate was partially withdrawn into the anterior chamber, from which the parasite was extracted through paracentesis (Fig. 3). The helminth was placed in a balanced saline solution and sent to a parasitology laboratory. Total vitrectomy with retinal revision was performed. The intraocular lens was implanted into the capsular bag. After the ports were removed, the surgery was completed.

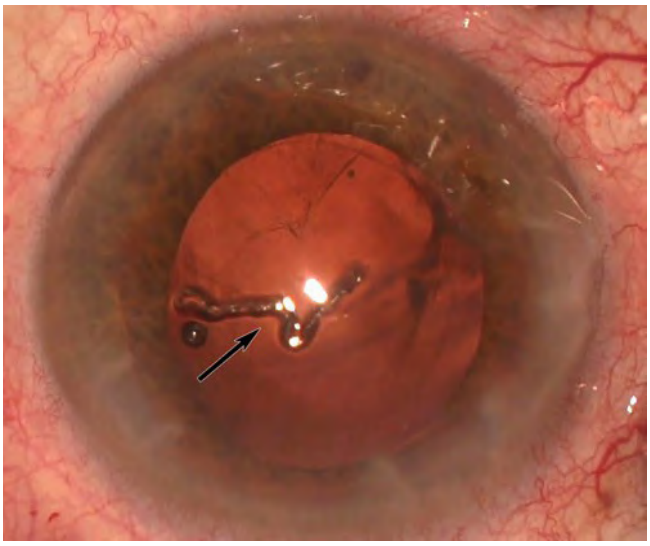

***FIG. 3.***
*Photoregistration of the surgical intervention stage: view of the worm located on the cornea after extraction from the vitreal cavity*

## RESULTS AND DISCUSSION

The postoperative period had no signs of an active inflammatory reaction. On the third day, the patient was discharged from the hospital. At the time of discharge, OS visual acuity was 0.8 and IOP was 10 mmHg. After 4 months, at the follow-up visit, OS visual acuity reached 1.0, and IOP remained at 10 mmHg. A female *Dirofilaria repens* was identified by parasitological examination. The patient is appointed a consultation with an infectious disease physician.

The peculiarity of this clinical case is the location of the patient's infection in an atypical area for this disease. The patient lives in the northern district of the Irkutsk region, beyond which she has not travelled for the last two years. Considering the timing of the complaints of decreased vision and the appearance of a mobile object in the field of vision, as well as the characteristics of the life cycle of the helminth, it can be assumed that

the infection occurred in the region of the patient's residence. This correlates with worldwide trends of a significant increase in the incidence of dirofilariasis in northern latitudes, which is associated with climatic warming and, consequently, with a longer life cycle of mosquito vectors of the disease, as well as the spread of stray cats and dogs, which are the final hosts of the parasite [14]. It is also worth noting that in one of the coldest and northernmost cities in Russia, Yakutsk, cases of dirofilaria infection in both domestic and wild animals have been revealed [13, 14].

Intraocular dirofilariasis is considered the rarest form of this disease and is sporadic. Surgery remains the generally accepted and the only treatment for this form of the disease. It is worth noting, however, that there is no consensus on the necessity of extracting the whole parasite and, if the parasite persists, the method of removing it from the eye. Some authors performing endovitreal intervention, simultaneously excise the dirofilaria, which significantly simplifies the intervention, reducing the duration of surgery. According to the literature, this method of parasite removal does not lead to the development of intraocular inflammation [6], but typing of the parasite in these cases becomes impossible. Domestic ophthalmologists follow the tactic of preserving the whole parasite, using various surgical approaches for this purpose. V.N. Kazaikin et al. described the technique of aspiration extraction of the parasite from the eye with preservation of the vitreous body [12]. R.R. Faizrakhmanov et al. used vitreotomy to mobilise the helminth with its further removal by forceps through a 23-gauge port [15]. In both cases, the integrity of the parasite was preserved for subsequent parasitological examination and for definitive diagnosis [12, 15].

The surgical treatment tactics used in this clinical case was conditioned by the presence of pronounced opacities in the crystalline lens, gross vitreous body opacities, which prevented adequate visualisation of the ocular fundus. In the postoperative period, the patient had visualised areas of pronounced chorioretinal atrophy of the peripheral retina, which may be a consequence of mechanical contact of the parasite or toxic effects of its products (Fig. 4). The absence of pathological changes in the macular region allowed to obtain high visual acuity.

Most publications that have presented the treatment of patients with this condition demonstrate obtaining high visual acuity without associated toxic and inflammatory reactions in the postoperative period. At the same time, clinical cases with catastrophic visual impairment caused by macular retinal atrophy as a consequence of prolonged presence of Dirofilariae in the eye have been described [16].

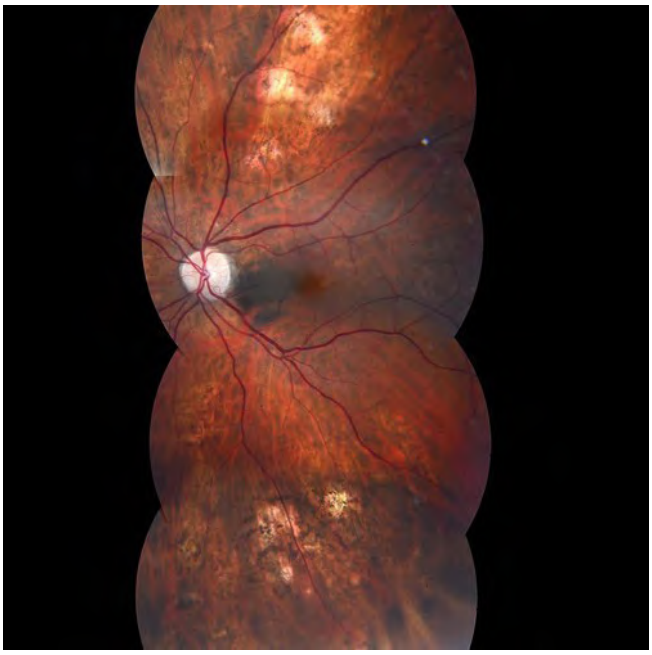

***FIG. 4.***
*Zones of pronounced chorioretinal atrophy of peripheral parts of the retina*

## CONCLUSION

This clinical case demonstrates the possibility of infection with the ocular form of dirofilariasis in a region atypical for the presence of this helminth. Despite the positive outcome of the disease, in the presented patient, the long-term presence of the parasite in the vitreal cavity led to the formation of chorioretinal atrophy in the peripheral retina, which confirms the need for timely diagnosis and surgical treatment.

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# SOME ASPECTS OF LABORATORY DIAGNOSTICS OF OPHTHALMODEMODECOSIS

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## ABSTRACT

*Demodicosis occupies a leading position among all dermatoses. According to ICD-10, it is not distinguished as a separate disease; it belongs to the class of parasitic diseases. The relevance of studying the problem of ophthalmodemodecosis is caused by its high prevalence and contagiousness, chronic course, an open issue of the role of the Demodex mite in the occurrence of inflammatory eye diseases, as well as the lack of effective methods for treating and preventing this pathology. In addition, Demodex causes discomfort and contributes to the occurrence of cosmetic defects, which in turn worsens the patient's quality of life.*

***The aim.*** *To present a review of the literature data and our own results of laboratory diagnostics of ophthalmodemodecosis.*

***Materials and methods.*** *The article presents clinical cases of demodectic eyelid lesions with different disease outcomes in case of similar treatment. Laboratory diagnostics included drawing up an acarogram. Epilated eyelashes were used as a material for detecting mites on eyelids. Counting of individuals was carried out using light microscopy; all forms of mite development were taken into account. The work presents the statistics on the frequency of examination of patients with suspected demodicosis at different times of the year, confirming the seasonality of this disease. The literature review included data on the history of studying the Demodex mite, existing hypotheses and theories about its pathogenesis, as well as the information on domestic and foreign methods of treating ophthalmodecosis, including modern hardware techniques.*

***Results.*** *Demodex mites play a significant role in the development of blepharitis and blepharoconjunctivitis. It is important to consider that demodicosis can occur against the background of inflammatory eye diseases of another etiology. Therapy for ophthalmodemodecosis currently remains complex, lengthy and ineffective. When assessing the results of an acarogram, any detected stages of a mite are clinically significant, and there isn't a direct relationship between the number of detected mites and the severity of clinical manifestations in all cases.*

***Key words:*** *Demodex, blepharoconjunctivitis, laboratory diagnostics, ophthalmodemodecosis, IPL therapy*

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# НЕКОТОРЫЕ АСПЕКТЫ ЛАБОРАТОРНОЙ ДИАГНОСТИКИ ОФТАЛЬМОДЕМОДЕКОЗА

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## РЕЗЮМЕ

*Демодекоз занимает лидирующее положение среди всех дерматозов. Согласно МКБ-10, он не выделяется как отдельное заболевание, относится к классу паразитарных болезней. Актуальность изучения проблемы офтальмодемодекоза обусловлена его высокой распространённостью и контагиозностью, хроническим течением, открытым вопросом о роли клеща Demodex в возникновении воспалительных заболеваний глаз, а также отсутствием эффективных методов лечения и профилактики данной патологии. Помимо этого, Demodex вызывает дискомфорт и способствует возникновению косметических дефектов, что в свою очередь ухудшает качество жизни пациента.*

***Цель работы.*** *Представить обзор литературных данных и собственных результатов лабораторной диагностики офтальмодемодекоза.*

***Материалы и методы.*** *В статье представлены клинические случаи демодекозного поражения век с различными исходами заболевания при аналогичном лечении. Лабораторная диагностика включала в себя составление акарограммы. Материалом для обнаружения клещей на веках служили эпилированные ресницы. Подсчёт особей осуществлялся методом световой микроскопии, учитывались все формы развития клеща. Приведена статистика частоты обследования пациентов с подозрением на демодекоз в различное время года с подтверждением сезонности данного заболевания. Литературный обзор включал в себя данные об истории изучения клеща рода Demodex, существующие гипотезы и теории о его патогенезе, а также информацию об отечественных и зарубежных методах лечения офтальмодемодекоза, включая современные аппаратные методики.*

***Результаты.*** *Клещ рода Demodex играет значительную роль в развитии блефаритов и блефароконъюнктивитов. Важно учитывать то, что демодекоз может возникать на фоне воспалительных заболеваний глаз другой этиологии. Терапия офтальмодемодекоза в настоящее время остаётся сложной, длительной и малоэффективной. При оценке результатов акарограммы клинически значимыми являются любые обнаруженные стадии клеща, а также не во всех случаях наблюдается прямая зависимость между количеством обнаруженных клещей и тяжестью клинических проявлений.*

***Ключевые слова:*** *Demodex, блефароконъюнктивит, лабораторная диагностика, офтальмодемодекоз, IPL-терапия*

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Inflammatory eye diseases of demodectic aetiology have a high prevalence worldwide *Demodex* mites are known to play a role in the development of various ophthalmic diseases, as well as complicate their course [1]. These diseases include blepharitis, blepharoconjunctivitis, chalazion, episcleritis, and marginal keratitis. According to domestic studies, asymptomatic carrier state occurs in a significant number of patients. The incidence of demodecosis is similar in men and women. In persons over 70 years of age, the probability of detecting a mite is almost 100 % [2].

Demodecosis is an infectious disease caused by the parasitisation of the opportunistic mite of the genus *Demodex*. The mite reproduces in hair follicles, sebaceous glands and mainly affects the face and external auricles, and rarely the skin of the chest and back [3].

The *Demodex* mite was first described as a worm by Jacob Henle in 1841. It was later correctly classified as the human mite *Acarus folliculorum* by dermatologist Carl Gustav Theodore Simon. In 1843 Richard Owen calls the mite *Demodex*. In the course of studies in 1970, L.H. Akbulatova discovered and described two forms of the mite, *Demodex folliculorum brevis* and *Demodex folliculorum longus*, – parasitising humans [4].

*Demodex folliculorum longus* has a long body and its predominant site of localization is hair follicles. The second form of *Demodex folliculorum brevis* has a shorter body and parasitizes the meibomian and Zeiss glands. The entire body of the mite is covered with a chitinous shell, legs are short, with the presence of fingernails at the ends (Fig. 1).

After fertilization, males die and females lay eggs in the cavity of hair follicles. The development cycle of the mite consists of five phases and lasts about 15–25 days. *Demodex* feeds on the secretion of sebaceous glands and the cytoplasm of epithelial cells. The viability of mites is not affected by low temperatures or low humidity, but at temperatures below 14 °C they fall into a state of torpor. They are viable in water for up to 25 days (at a temperature of 12–15 °C), in pus and dead skin layers – 20 days, in dry air – 36 hours. *Demodex* mites are most active at temperatures of 30–40 °C, and at high temperatures (53–55 °C) they die after 18 days. In cosmetic cream, vegetable oil, petroleum jelly mites retain their vital activity for a long time [5].

There are several main hypotheses regarding the pathogenicity of the *Demodex* mite. Some authors believe that the mite manifests its pathogenicity by transporting microorganisms into the sebaceous glands and hair follicles. As an example, *Bacillus oleronius*, found on the tick surface, can activate both the ticks themselves and other microorganisms, and its proteins cause an increase in pro-inflammatory cytokines (interleukin (IL) 6 and IL-1b).

The following hypothesis is based on the fact that mites become active under the influence of endogenous and exogenous factors that contribute to a decrease in immunity (diabetes mellitus, gastrointestinal diseases, thyroid disorders, cardiovascular diseases, bad habits and stress) [6].

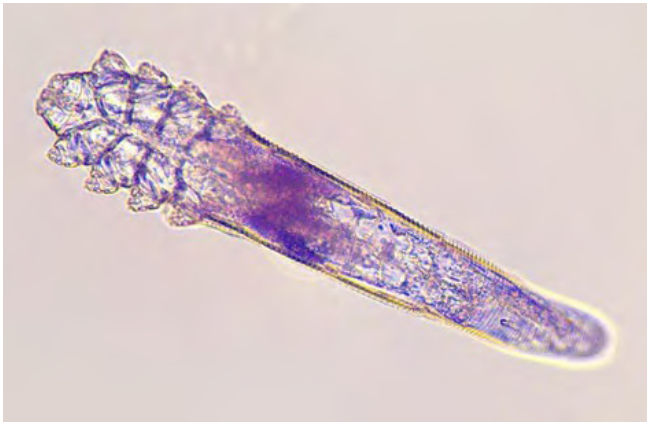

***FIG. 1.***
*Demodex folliculorum longus mite. Photo was taken using Lomo MC-8.3 digital camera (Lomo JSC, Russia); magnification × 400*

Foreign authors cite data reflecting the link between the rise in incidence in the spring and summer period, which is explained by increased synthesis of cathelicidin molecules supporting the inflammatory process (LL-37-natural antimicrobial barrier) as a result of the body's production of vitamin D under the influence of ultraviolet radiation [7].

According to the data of studies carried out in the Irkutsk branch of the Federal State Autonomous Institution «S.N. Fedorov Eye Microsurgery Centre» of the Ministry of Health of Russian Federation, it can also be observed that clinical manifestations of demodecosis have their seasonality (Fig. 2).

*Demodex* mites and their waste products cause chemical and mechanical irritation. With their jaws (chelicerae) they destroy skin cells. This leads to inflammation in the form of infiltrates and deposition of keratin protein and lipids in the stratum corneum. According to Yu.N. Koshevenko (2008), as a result of mite vital activity, the accumulation of metabolic products creates conditions for the attachment of various infections [8].

A.Ya. Varapetov (1972) asserts that «one of the results of mite activity is stagnation of sebum, which leads to prolonged irritation of the nerve-receptor apparatus of the sebaceous-hair follicle» [9].

Following another theory, mites of the genus *Demodex* are capable of inducing various immune responses. In the scientific literature, there is evidence of violations of the cellular link of immunity, manifested in a decrease in phagocytosis and expression of $CD3^+$, $CD4^+$, $CD8^+$, increased production of $CD22^+$, IgM, tumor necrosis factor α, IL-4, IL-6, increased phagocytosis and expression of $CD25^+$, $CD95^+$, HLA-DR+ [10].

There are studies upon which the theory of genetic predisposition to demodecosis disease is based [11].

To date, there is no consensus on the etiology and pathogenesis of the mite genus *Demodex*.

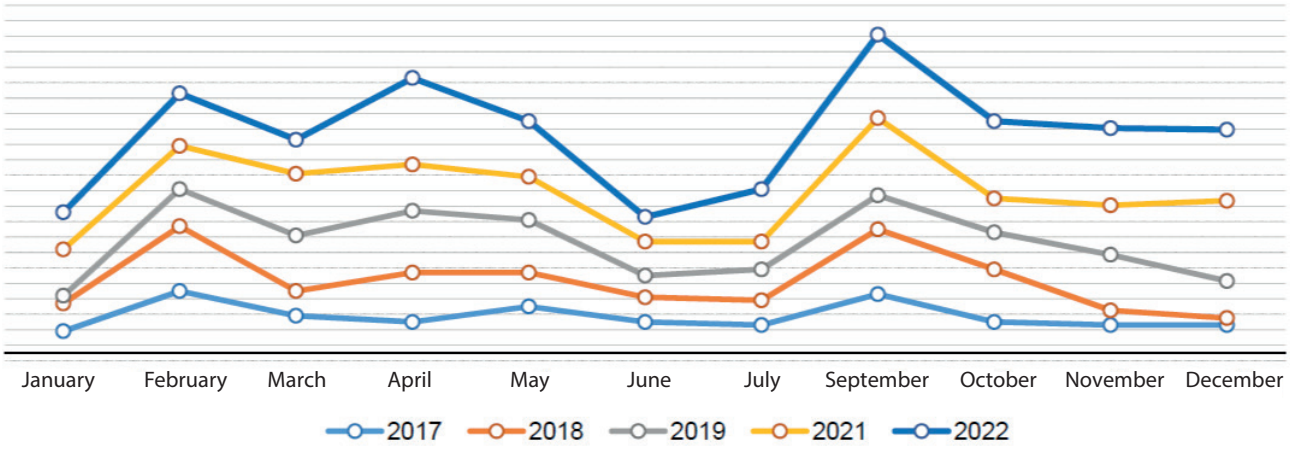


***FIG. 2.***
*Distribution of the number of demodecosis laboratory tests (acarograms) during the year*

All existing hypotheses and theories are contradictory and diverse.

In 1979, N.D. Zatsepina proposed the following classification of demodecosis of the visual organ: «asymptomatic carrier, obliterated forms, demodecosis blepharoconjunctivitis (uncomplicated, complicated), demodecosis episcleritis, demodecosis keratitis, demodecosis iridocyclitis» [12]. It is known that ophthalmodemodecosis can either act as an independent disease or be combined with demodecosis of other parts of the body.

At an ophthalmologist's appointment, patients have the following complaints: a feeling of a «foreign body» in the eyes, periodic discomfort, rubbing, itching of the eyelids, sometimes eyebrows, thick discharge in the corners of the eyes [13]. When examining the patient, the following is observed: eyelid margins are thickened, hyperaemic, meibomian gland ducts are filled with thick secretion, superficial plaque in the form of «muffs» at the root of the eyelashes is observed [14].

Only after laboratory diagnosis can a diagnosis of demodecosis be confirmed. For 1 day before the research the patient is recommended not to use decorative and medicinal cosmetics, as well as if possible not to use eye drops. The material for the study is epilated eyelashes, 4 pieces from each eyelid. This material is placed on a slide, in a drop of glycerol, covered with a coverslip and examined in a light microscope at a magnification of ×100.

The identification of the parasite is based on an acarogram by counting the different developmental stages (larvae, eggs and adults) (Fig. 3).

Acarogram is an objective diagnostic criterion. The presence of more than 4 specimens in the preparation is an indication of mite activity. As clinical practice demonstrates, large numbers of mites may be found on the eyelid margins in the obliterated clinic of blepharitis, and, conversely, in the vivid course of the disease that requires treatment, mites may be detected in small numbers.

The course of treatment for ophthalmodemodececosis may last 30–40 days, followed by retreatment in 2–3 months. The three-layer cuticle, which densely covers the surface of the *Demodex* mite, creates an obstacle to the action of the preparations.

Today the main method of blepharitis treatment in Russia is a complex application of anti-inflammatory, antibacterial, antiparasitic therapy with therapeutic eyelid hygiene and the use of tear substitutes.

Anti-inflammatory therapy is used to eliminate swelling and hyperaemia. Corticosteroid ointments, by reducing local immunity, increase the number of mites, so their use is not recommended [15].

Therapeutic eyelid hygiene is important in the treatment of ophthalmodemodecosis. This method of treatment was proposed by Professor G.S. Polunin in 2007 [16]. Three-stage eyelid hygiene is carried out: first, warm compresses improve metabolism; eyelid massage allows the evacuation of viscous secretions; then the eyelid margins are treated with disinfectants and acaricides.

Nowadays, there is a large number of modern devices for eyelid heating, moisturising and massage on the world market: in Russian ophthalmological practice, the most widely used are the Blephasteam mask [17] and the LipiFlow system, with the help of which the simultaneous effect on the inner surface of the upper and lower eyelid by heat and pulsating mechanical pressure is carried out.

In Europe, Optima IPL is used to treat dry eye disease and restore meibomian gland function. In 2002, V.G. Prieto et al. found that IPL (intense pulsed light)

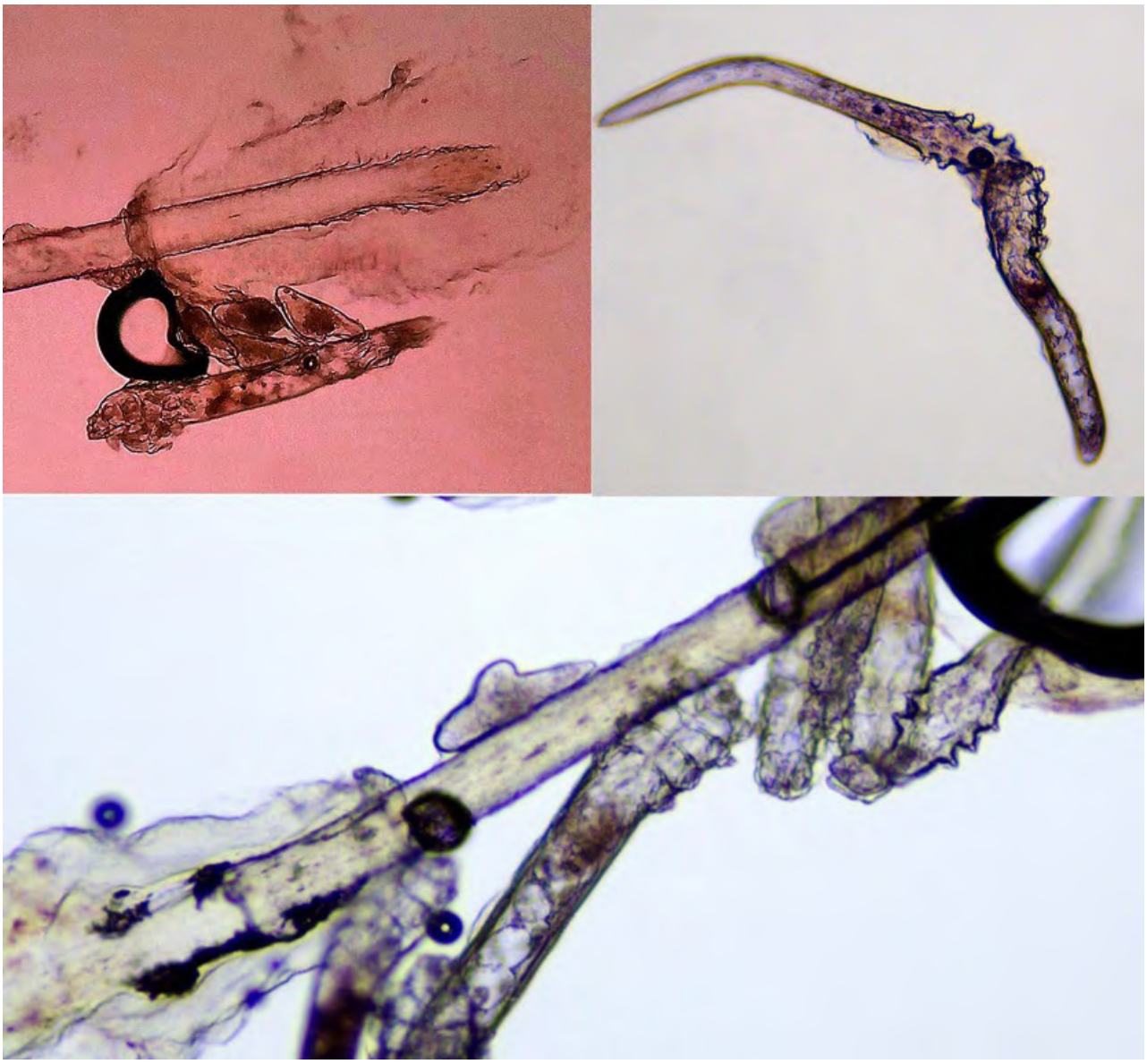


***FIG. 3.***
*Developmental stages of the mite Demodex folliculorum longus: а – adult and 4 eggs; б – adult and larva; в – adult, larva and egg (magnification ×100)*

therapy resulted in coagulation necrosis of *Demodex* mites and reduction of perifollicular lymphoid infiltrate [18]. Dr. Rolando Toyos has perfected a specific treatment protocol that maximises the long-term elimination of the *Demodex* mite [19].

After a month from the beginning of treatment a control acarogram is carried out, and in the absence of dynamics or an increase in the number of specimens, antiparasitic (acaricidal) drugs are used. Chloroform, carbolic acid, tar, cresol, ether and dichlophos are known to kill *Demodex* instantly. Preparations based on these substances are used in veterinary medicine and dermatology, but are not used in ophthalmology due to their toxicity. Metronidazole has proven to be highly effective over the years. The treatment of demodecosis with metronidazole, however, has not been successful in recent years [20]. Ornidazole may also be the drug of choice.

There are many data about the successful use of fluoroquinolones in the treatment of blepharitis [21]. Additionally, for the purpose of antibacterial therapy, benzildimethyl-myristoylamino-propylammonium-based preparations are prescribed as instillations into the conjunctiva [22].

It has also been observed that the consequence of impaired outflow of meibomian gland secretion is a decrease in the lipid layer of the tear film, which accelerates its evaporation and causes the development of «dry eye» syndrome [23]. The use of multicomponent lacrimal substitute preparations (LSPs), which include carmellose and hydroxypropylguar, has an advantage over the use of single-component LSPs, which has been statistically proven [24]. Alcoholic solutions of calendula, wormwood, tea tree oil, which have antimicrobial and antiseptic effect, are of great importance in the treatment of blepharitis of demodectic etiology.

It has been observed that cholinomimetics used in the treatment of glaucoma paralyses the musculature of mites due to muscarinic and nicotine-like action. Such drugs include 0.02 % phosphacol, physostigmine, 0.5 % tosmilene, 0.01 % armin [2].

The *Demodex* mite does not tolerate an alkaline environment and therefore alkaline eye drops and zinc sulphate drops in boric acid are used as symptomatic treatment.

Sanitation of foci of infection, treatment of concomitant diseases, limitation of sun exposure and dietary intake of polyunsaturated fatty acids such as Omega-3 are important in the treatment of ophthalmodemodecosis [25].

The above presented modern approaches to the diagnosis and treatment of ophthalmodemodecosis can be demonstrated by clinical cases.

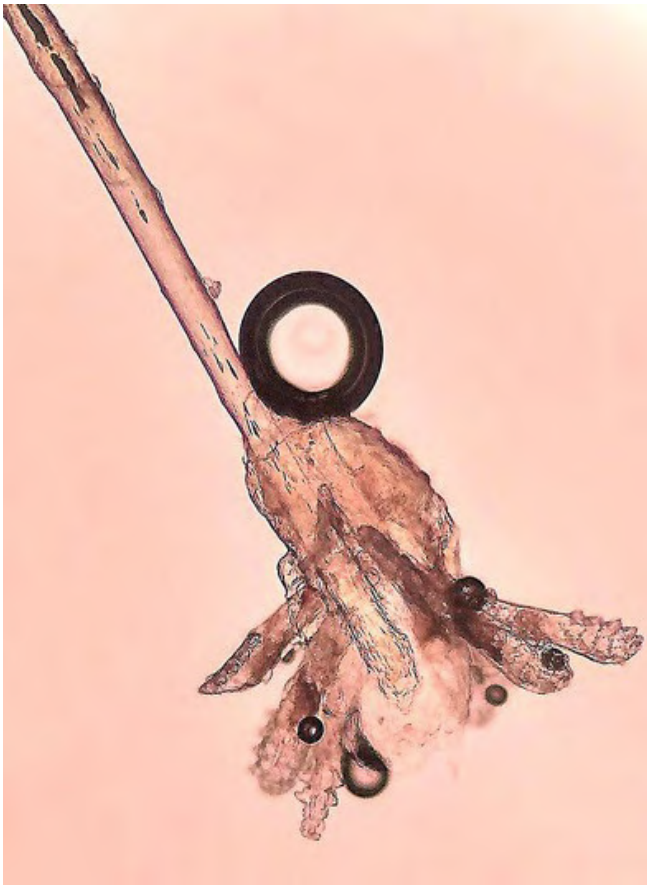

***FIG. 4.***
*Demodex folliculorum longus mites. Magnification ×100; lens 10/0.25; eyepieces WF 10×/22*

**Clinical case No. 1**

Patient L., 66 years old, complained of decreased near vision, dryness, discomfort, and itching of the eyelids of both eyes.

Biomicroscopy revealed the following: intermarginal eyelid margins thickened; meibomian glands filled with their own content. There are small gray muffs on the eyelashes.

The patient was examined for demodecosis; an acarogram revealed 8 adult specimens, 1 larva and 1 egg (Fig. 4).

Diagnosed with chronic demodectic blepharitis in both eyes.

Treatment was prescribed: eyelid massage No. 1–2; washing with tar soap; taking Trichopol; treatment of eyelid margins with Blepharogel-2, calendula alcohol solution. Oxyal drops (or Chilo-comod, Thealoz) 1 drop 3 times a day in both eyes for 1 month, then – as needed. Taking vitamin preparations with lutein, zeaxanthin, Omega-3, 6, 2 courses per year. Wearing sunglasses.

The patient came for a follow-up examination 2 months later. Acarogram results revealed 4 adults, 2 larvae and 1 egg.

Continuation of treatment was recommended; consultation with a dermatologist in the place of residence.

The patient came for a follow-up examination 2 months later.

Subjectively: after the treatment she observed improvement – disappearance of itching of eyelids of both eyes.

Objectively: eyelid skin without peculiarities; no *Demodex* mites were found according to the results of acarogram.

**Clinical case No. 2**

Patient V., 49 years old, complained of low vision of both eyes in the distance, discomfort, itching of the eyelids of both eyes. Visited a dermatologist at the place of residence for treatment of acne.

Concomitant diseases: euthyroidism.

When examined, the intermarginal margins of the eyelids are thickened.

The patient was tested for demodecosis; an acarogram revealed 18 adult specimens, 8 larvae and 7 eggs (Fig. 5).

Diagnosed with demodectic blepharitis in both eyes.

Treatment was prescribed: Stillavit drops (or Systein Ultra, Vizmed) 1 drop 3 times a day in both eyes for 1 month, further – as the circumstances require. Trichopol reception, eyelid treatment with Blepharolosione, Blepharogel-2. Re-examination after the course of treatment.

***FIG. 5.***
*Demodex folliculorum longus mites. Photo taken using Lomo MC-8.3 digital camera (Lomo JSC, Russia); magnification ×100; objective 10/0.25; eyepieces WF 10×/22*

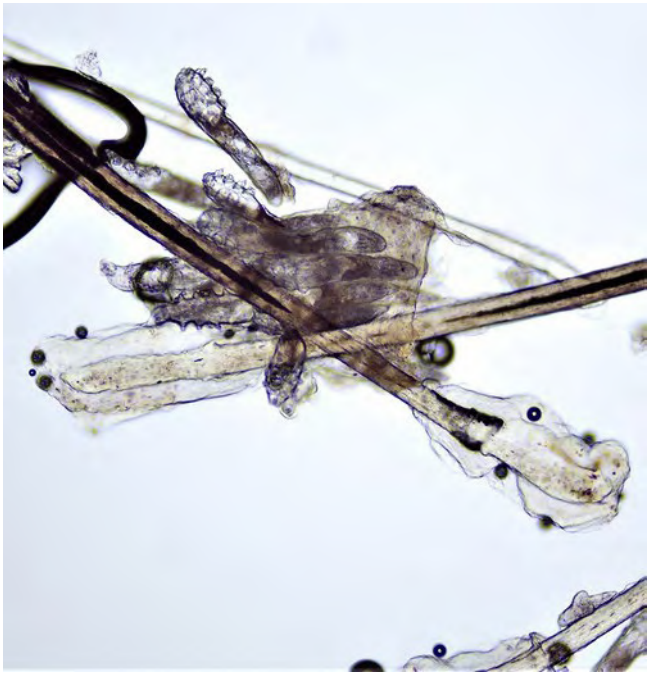

***FIG. 6.***
*Demodex folliculorum longus mites (adults, larva and egg). Photo taken using Lomo MC-8.3 digital camera (Lomo JSC, Russia); magnification ×100; objective 10/0.25; eyepieces WF 10×/22*

The patient came for a follow-up examination 1 month later.

Subjectively: observes a decrease in complaints.

Objective: intermarginal margins of eyelids thickened.

A repeat acarogram revealed 21 adults, 1 larva and 1 egg.

It is recommended to continue local treatment of demodectic blepharitis.

The patient came for a follow-up examination 3 months later.

Subjective: observes disappearance of complaints.

Objectively: eye adnexa without peculiarities.

Acarogram result: 5 adults and 1 larva were revealed.

**Clinical case No. 3**

Patient N., 49 years old, complained of insufficient vision in both eyes, periodic redness and peeling of the eyelid skin.

Examination: eyelid skin moderately hyperemic, single scales on eyelashes.

Acarogram result: 5 specimens were revealed.

Treatment of demodecosis was prescribed at the place of residence: heat compresses on eyelid skin 2 times a day for 2 months; Blefarolotion – treatment of eyelid skin edges 2 times a day for 2 months; Blefarogel-2 – treatment of eyelid skin edges 2 times a day for 2 months.

The patient came for a follow-up examination 2 months later.

Acarogram result: 14 specimens were revealed (Fig. 6).

Trichopol 0.25 mg regimen for 1 month was added to the treatment.

The patient came in after treatment for demodecosis for a follow-up examination 2 months later.

Examination: eyelid skin without peculiarities.

Acarogram result: 16 specimens were revealed.

Continued treatment was recommended.

The patient came for a checkup 4 years later.

Examination: eyelid skin pink, single «muffs» on eyelashes.

Acarogram result: 19 specimens were revealed.

Therefore, despite the ongoing therapy, no positive dynamics was obtained.

## CONCLUSIONS

The data presented in this review suggest that despite the wide variety of drugs and cosmetics available for the treatment of ophthalmodemodecodosis, therapy remains difficult, time-consuming, and ineffective.

In treatment, in an attempt to treat the problem in a holistic manner, it is worth avoiding the abundance of prescribed medications, as this reduces patient adherence to treatment.

In assessing acarogram results, any mite stages that were found are clinically significant.

The use of ointments, tear-replacement drugs can aggravate the course of the disease.

The importance of *Demodex* mites in the development of blepharitis and blepharoconjunctivitis should not be underestimated, and it should be considered that demodecosis may occur against the background of inflammatory eye diseases of other etiologies.

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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## PSYCHOLOGY AND PSYCHIATRY

# PSYCHOMETRIC PROPERTIES OF THE ABBREVIATED MATH ANXIETY SCALE ON A SAMPLE OF RUSSIAN HIGH SCHOOL STUDENTS

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## ABSTRACT

***Background.*** *Math anxiety is a state of fear and anxiety that an individual experiences when interacting with mathematical problems. Currently, there is a lack of questionnaires to measure mathematical anxiety for Russian-speaking schoolchildren.*
***The aim.*** *The study analyzed the factor structure and psychometric properties of the Abbreviated Math Anxiety Scale (AMAS).*
***Materials and methods.*** *The study involved 1,198 schoolchildren in grades 10–11. The psychometric properties of the AMAS were analyzed.*
***Results.*** *AMAS demonstrated bifactor structure: subscales of Learning Math Anxiety (LMA) and Math Evaluation Anxiety (MEA) and general scale of Math Anxiety. The bifactor model demonstrated the best fit indices. Analysis confirmed reliable internal consistency (Cronbach's alphas for LMA = 0.82, MEA = 0.75, total AMAS = 0.95). External validity of AMAS has been confirmed. LMA showed lower scores than MEA. The distribution of scores on the general AMAS scale was shifted to low values. Girls showed higher scores on all scales of the questionnaire. The analysis also confirmed measurement invariance for both boys and girls.*
***Conclusion.*** *Based on the analysis, we can conclude that the AMAS is a valid tool for assessing mathematical anxiety in high school students.*

***Key words:*** *math anxiety, Abbreviated Math Anxiety Scale, factor validity, psychometric properties, high schoolers*

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# ПСИХОМЕТРИЧЕСКИЕ СВОЙСТВА СОКРАЩЁННОЙ ШКАЛЫ МАТЕМАТИЧЕСКОЙ ТРЕВОЖНОСТИ НА ВЫБОРКЕ РОССИЙСКИХ СТАРШЕКЛАССНИКОВ

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## РЕЗЮМЕ

***Обоснование.*** *Математическая тревожность – это состояние страха и тревоги, которое испытывает индивид, когда взаимодействует с математическими задачами. В настоящее время имеется недостаток опросников для измерения математической тревожности у русскоговорящих школьников.*

***Цель исследования.*** *В исследовании анализировались факторная структура и психометрические свойства Сокращённой шкалы математической тревожности (СШМТ).*

***Методы.*** *В исследовании приняли участие 1198 школьников 10–11-х классов. Производился анализ психометрических свойств по СШМТ.*

***Результаты.*** *Анализ СШМТ выявил бифакторную структуру опросника с субшкалами тревожности изучения математики (ТИМ) и тревожности математической оценки (ТМО) и общей шкалой математической тревожности. Бифакторная модель продемонстрировала лучшие индексы соответствия. Анализ подтвердил надёжные оценки внутренней согласованности (альфа Кронбаха для субшкалы ТИМ = 0,82, для субшкалы ТМО = 0,75, для общей СШМТ = 0,95). Была подтверждена внешняя валидность СШМТ. ТИМ обнаружила меньшие оценки по сравнению с ТМО. Распределение оценок общей шкалы СШМТ было смещено к низким значениям. Девочки продемонстрировали более высокие показатели по всем шкалам опросника. Анализ также подтвердил инвариантность измерения как для мальчиков, так и для девочек.*

***Заключение.*** *На основе проведённого анализа можно сделать вывод, что СШМТ является валидным инструментом для оценки математической тревожности у старшеклассников.*

***Ключевые слова****: математическая тревожность, Сокращённая шкала математической тревожности, факторная валидность, психометрические свойства, старшеклассники*

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## INTRODUCTION

Mathematical anxiety (MA) is a state of fear and worry that a person experiences when solving problems related to mathematics. Possible definitions are provided by Lazarus, Hembree, Ashcraft and Faust, and other authors [1].

### The nature of math anxiety and math anxiety theories

Sources of MA can be found in attitudes, upbringing, and school traumas. Twin studies show that environmental factors explain up to 70 % of math anxiety [2, 3], among them such as: parents' fear of mathematics, low expectations from the child, bad school experience, gender stereotypes and other related reasons [4–12].

The theory of mechanisms of insufficient inhibition [8] describes the MA feedback loop: first, the MA is triggered by a mathematical problem; then, when an individual begins to experience anxiety, it limits their working memory and impairs their ability to solve mathematical problems, which in turn leads to incorrect solving of a mathematical problem. In other words, MA primarily affects working memory, and people who exhibit high levels of MA devote some of their working memory capacity to rumination about their anxiety. EEG source localization using specialized approaches (in particular, sLORETA) shows that brain regions such as the insula and amygdala are activated in people with MA. The insula is involved in reactions to pain, and the amygdala is associated with emotional experiences (fear, stress, anxiety). These data support the theory that explains MA by the anticipation of pain [9]. The attentional control theory of anxiety posits that anxiety reduces the effective functioning of attention and increases attention to threat-related stimuli [10]. Thus, individuals with high MA demonstrate reactive (post factum) control, whereas individuals with low anxiety demonstrate proactive (anticipatory) control.

### Math anxiety and test anxiety

Since mathematics is an academic subject that is taught in most schools and universities, when studying it, schoolchildren and students inevitably face the situation of assessing their knowledge in this course. Assessment of mathematical knowledge is associated with so-called test anxiety – stress associated with passing a test, often leading to a decrease in performance (as defined by the American Psychological Association Dictionary). The concept of test anxiety began to develop in the middle of the 20th century [11]. Test anxiety has a negative impact on academic achievement and test scores. However, in the Russian context there is no instrument for measuring the level of test anxiety of students, and the terms "test anxiety" and "exam stress" are used as synonyms. When measuring MA, it is important to have a tool that can differentiate it itself from evaluation-related test anxiety. This is especially true for scales that are developed for older groups of students, since they often find themselves in knowledge assessment situations and demonstrate test anxiety.

### Sex differences and gender stereotypes in the development of math anxiety

Studies show increased MA levels in girls [12]. This may be due to the influence of gender stereotypes: girls are often told that they are less mathematically inclined than boys. This influences girls' attitudes towards mathematical activities. On the other hand, some studies also report that instead of math anxiety, girls may be affected by test anxiety [13]. Thus, girls may experience more stress in a math test situation. Some studies report that in adolescence, girls begin to rate their mathematical abilities lower than boys, which can be explained by the increasing influence of gender stereotypes with age [14].

### The Abbreviated Math Anxiety Scale

The Abbreviated Math Anxiety Scale (AMAS) was developed by D.R. Hopko and colleagues [15]. The scale is designed to measure MA. Each task describes a situation associated either with the process of learning mathematics or with the process of testing mathematical knowledge. Participants are required to report the level of their anxiety in the described situation on a five-point scale. Answers range from "low" to "high" anxiety. Filling out the form takes no more than 5 minutes. This scale was originally developed for undergraduate students. It consists of 9 items and includes two subscales: LMA (learning math anxiety) and MEA (math evaluation anxiety). The ability to measure both components of MA (in the situation of learning mathematics and assessing mathematical knowledge) is a noticeable advantage of this technique. At the same time, the two-factor structure is not the only one possible based on the results of analysis in different countries. Some authors report a two-factor configuration, others report a different structure, particularly with one question belonging to two scales [16–19]. The test has high internal consistency ($\alpha = 0.90$) and reliability (two-week period, $r = 0.85$) [15]. Convergent validity of the AMAS was demonstrated using the MARS-R (the Mathematics Anxiety Rating Scale) and reached $r = 0.85$. In Russia, the AMAS has not previously been tested among high school students. Validation of the AMAS will allow the use of a questionnaire that separates MA itself and anxiety associated with assessing mathematical knowledge, which is important for high school students who are constantly faced with exams and tests in mathematics. This will allow to evaluate the differences between the two components of MA in boys and girls. Thus, the purpose of this study is to confirm and evaluate such psychometric properties of the AMAS as factorial validity, internal consistency, external validity, and measurement invariance between genders in a sample of high school students in grades 10–11. Research on MA in general and its diagnostics in particular is an important step in the prevention of anxiety disorders characterized by significant and uncontrollable experiences of anxiety and fear, such that a person's social, professional and personal functions are disrupted. Our study contributes to public health by validation of MA scale aimed at early detection of MA. This investigation will help specialists to prevent the neg-

ative consequences of MA on the mental health and well-being of adolescents.

## MATERIALS AND METHODS

### Sample

Initially, 2,409 participants completed the questionnaire. The following were removed from the sample:

- those who specified their age as lower than 12 y. o.;
- those who didn't identify their sex;
- those who took less than 100 ms to complete the test;
- those who took more than 1,500 ms to complete the test;
- those with Mahalanobis distance *p*-value less than 0.001.

Exclusion of those who reported their age below 12 is because of the implausibility of such age for high school students. Exclusion of who did not specify their gender is due to the impossibility of including them in the analysis of gender differences. The exclusion of those who completed the test in less than 100 ms or more than 1,500 ms, as well as those with Mahalanobis distance p-value less than 0.001, is due to a high probability of poor questionnaire completion and, consequently, unreliable data [20, 21].

Thus, the final sample consisted of 1,198 schoolchildren. Age ranged from 15 to 18 years (mean = 16.52; median = 17.0; standard deviation = 0.63). Among respondents 502 (42 %) were male, 696 (58 %) were female. The study included grades 10 and 11. There were 583 children in grade 10 and 615 were in grade 11.

### Procedure

The study was conducted online. Informed consent was obtained from the parents of all participants. The study was approved by the Ethics Committee of the Psychological Institute of the Russian Academy of Education (protocol No. 2020/4-1 of April 2, 2020).

### Scales

*The Abbreviated Math Anxiety Scale* (AMAS) consists of 9 items. The AMAS was adapted from the work of D.R. Hopko et al. [15]. This version has been translated into Russian by professional translators (Table 1). Translation, reverse translation and adaptation of the test were performed by the Cognitive and Interdisciplinary Research Laboratory (Sirius). The questionnaire consists of 9 items: 5 items for the Learning Math Anxiety scale and 4 items for the Math Evaluation Anxiety scale. Respondents were asked to rate each statement in terms of how anxious they felt in each of the situations described. Responses were given on a Likert scale from 1 (low anxiety) to 5 (high anxiety).

*State-Trait Anxiety Inventory STAI-T* (Scale of Personal Anxiety trait) [22]. This scale is one of the scales of State-Trait Anxiety Inventory (STAI-Scale) of Reactive and Personal Anxiety by Spilberger. The STAI-T assesses relatively stable aspects of anxiety proneness, including general states of calm, confidence, and security. Participants need

**TABLE 1**

**ABBREVIATED MATH ANXIETY SCALE IN ENGLISH AND RUSSIAN**

| Item No. | English | Russian |
|---|---|---|
| 1 | Having to use the tables in the back of a math book. | Используя таблицы в конце учебника по математике. |
| 2 | Thinking about an upcoming math test 1 day before. | Думая накануне о предстоящем тесте по математике. |
| 3 | Watching a teacher work an algebraic equation on the blackboard. | Наблюдая, как преподаватель объясняет алгебраическое уравнение на доске. |
| 4 | Taking an examination in a math course. | Выполняя экзамен по математике. |
| 5 | Being given a homework assignment of many difficult problems that is due the next class meeting. | Получая домашнюю работу с большим количеством трудных задач, которую нужно решить к следующему занятию. |
| 6 | Listening to a lecture in math class. | Слушая лекцию на занятии по математике. |
| 7 | Listening to another student explain a math formula. | Слушая, как другой студент объясняет математическую формулу. |
| 8 | Being given a "pop" quiz in math class. | Выполняя внеплановую контрольную на занятии по математике. |
| 9 | Starting a new chapter in a math book. | Начиная новую главу по математике. |

**Note.** Instruction for participants: please rate each statement in terms of how much anxiety you feel in each of the situations described. Response scale: 1 – low anxiety; 2 – slight anxiety; 3 – moderate anxiety; 4 – major anxiety; 5 – high anxiety.

to rate how they usually feel. Ratings range from "almost never" to "almost always".

*Perceived Difficulty of Math (scale of Questionnaire "Gender stereotypes and incremental beliefs about STEM")* [23]. The scale is aimed at identifying difficulties associated with learning mathematics, according to self-reports of schoolchildren. This scale includes 4 items ("I usually do well in math (reverse coded)", "Math is harder for me than for many of my classmates", "Studying math gives me anxiety", "Math is harder for me than other subjects"). Participants rated all items on the same 4-point Likert scale (with 2 negative and 2 positive ratings). Cronbach's alpha (0.8) for the scale demonstrates good internal consistency.

### Data analysis

The first stage of analysis included the calculation of descriptive statistics. Confirmatory factor analysis (CFA) was then conducted using the WLSMV estimator. The following fit indices were used to evaluate the model: Root Mean Square Error of Approximation (RMSEA), Comparative Fit Index (CFI), Tucker – Lewis Index (TLI), and Standardized Root Mean Square Measure (SRMR). The next step in the analysis was to assess internal consistency using Cronbach's alpha. External validity was measured using Spearman correlations of the AMAS scales and other questionnaires. Differences between sexes were then assessed by comparing means. In addition, measurement invariance across genders was analyzed using structural equation modeling (SEM), a procedure that selected the "automatic" estimator in SEM offered in JASP. Measurement invariance was assessed using two measures: configural and metric invariance. Configurational invariance was assessed by the CFI and RMSEA indices. Metric invariance was assessed using ΔCFI and ΔRMSEA. All statistical measurements were performed in R version 4.2.1 and JASP.

## RESULTS

### Descriptive statistics

At the first stage of the analysis descriptive statistics were calculated. The results are presented in Table 2. As can be seen, LMA is less pronounced than MEA.

**TABLE 2**

**DESCRIPTIVE STATISTICS FOR LMA, MEA AND AMAS_TOTAL**

| Scales | Number of items | Mean | Std | Median | Max |
|---|---|---|---|---|---|
| LMA | 5 | 2.0 | 3.3 | 0.0 | 20 |
| MEA | 4 | 6.8 | 4.0 | 7.0 | 16 |
| AMAS_total | 9 | 8.8 | 6.3 | 8.0 | 36 |

Distribution graphs of the AMAS total scale and two subscales are presented in Figure 1. In general, the distribution of the AMAS is shifted towards low values. The obtained result is one of the typical variants of the distribution of MA scores: it has been shown that low MA scores are most common in the population [24]. Pearson's correlation between LMA and MEA is 0.51.

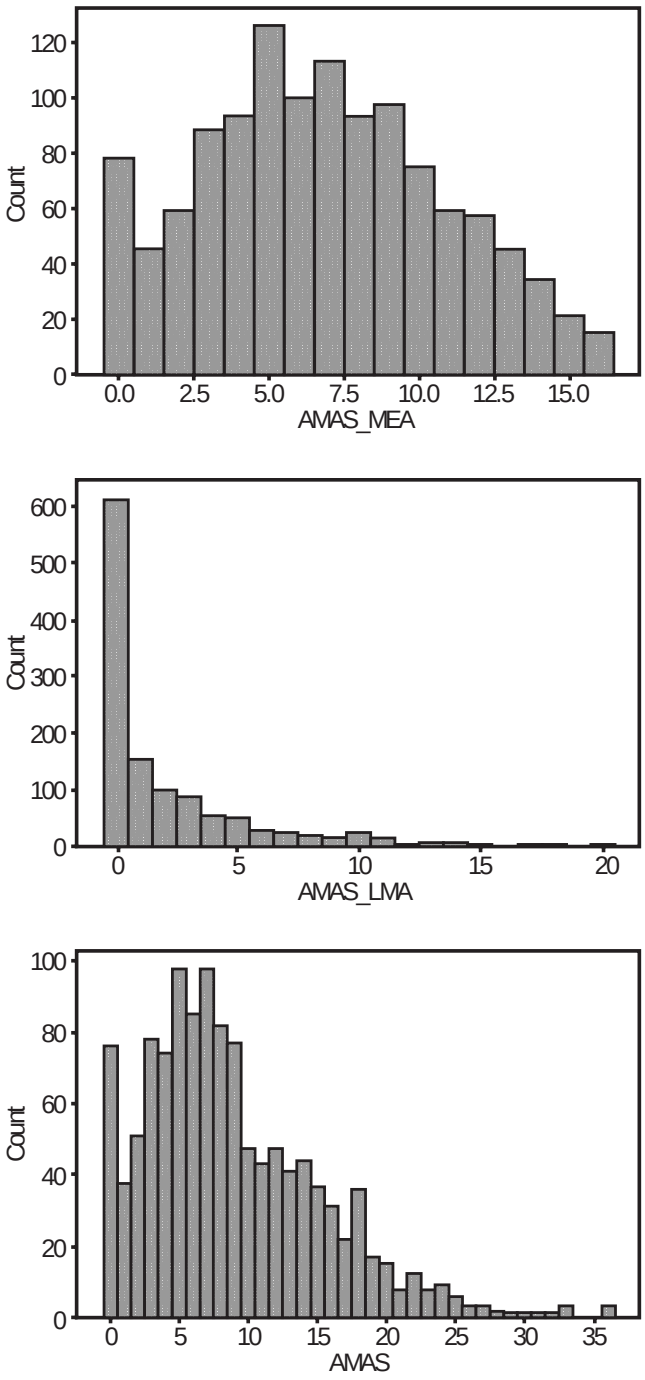


***FIG. 1.***

*Frequency histograms for LMA, MEA and total AMAS scales*

### Confirmatory factor analysis (CFA)

The CFA tested three models: one-factor, two-factor, and bifactor. The following indicators were used as standards in evaluating model fit indices: Standardized Root Mean Square (SRMR) < 0.08, Tucker – Lewis index (TLI) scores approaching 1, Comparative Fit Index (CFI) > 0.95, and Root Mean Square Error of Approximation (RMSEA) < 0.08. The bifactor model demonstrated good statistics and had better fit indices than the others: RMSEA = 0.018, CFI = 1.000, TLI = 0.999, SRMR = 0.023 (Table 3).

**TABLE 3**

**MODEL FIT INDICATORS (CONFIRMATORY FACTOR ANALYSIS, CFA)**

| Models | CFI | TLI | RMSEA | SRMR |
|---|---|---|---|---|
| 1-factor | 0.972 | 0.962 | 0.143 | 0.115 |
| 2-factor | 0.995 | 0.993 | 0.062 | 0.053 |
| Bifactor | 1.000 | 0.999 | 0.018 | 0.023 |

Standardized CFA loadings for the bifactor model are presented in Table 4.

Factor configuration of our data appropriates two factors of Learning Math Anxiety and Math Evaluation Anxiety with general (common) Math Anxiety Factor. LMA includes 5 items (1, 3, 6, 7, 9) MEA – 4 items (2, 4, 8, 5). The factor model is presented on Figure 2.

**TABLE 4**

**STANDARDIZED CFA LOADINGS FOR THE BIFACTOR MODEL**

| Item | Estimate | | |
|---|---|---|---|
| | Learning Math Anxiety | Math Evaluation Anxiety | General Math Anxiety |
| 1 | 0.279 | – | 0.647 |
| 3 | 0.306 | – | 0.825 |
| 6 | 0.469 | – | 0.777 |
| 7 | 0.500 | – | 0.704 |
| 9 | 0.290 | – | 0.791 |
| 2 | – | 0.518 | 0.704 |
| 4 | – | 0.675 | 0.519 |
| 8 | – | 0.584 | 0.685 |
| 9 | – | 0.246 | 0.592 |

### Internal consistency

To assess internal consistency, Cronbach's Alpha was analyzed and demonstrated the following: 0.82 for LMA, 0.75 for MEA, 0.92 for the total MA scale. The obtained alpha values above 0.7 showed good internal consistency and confirmed the internal consistency of the data.

### External validity

One of the possible ways to test the external (construct) validity of the AMAS may be to use school anxie-

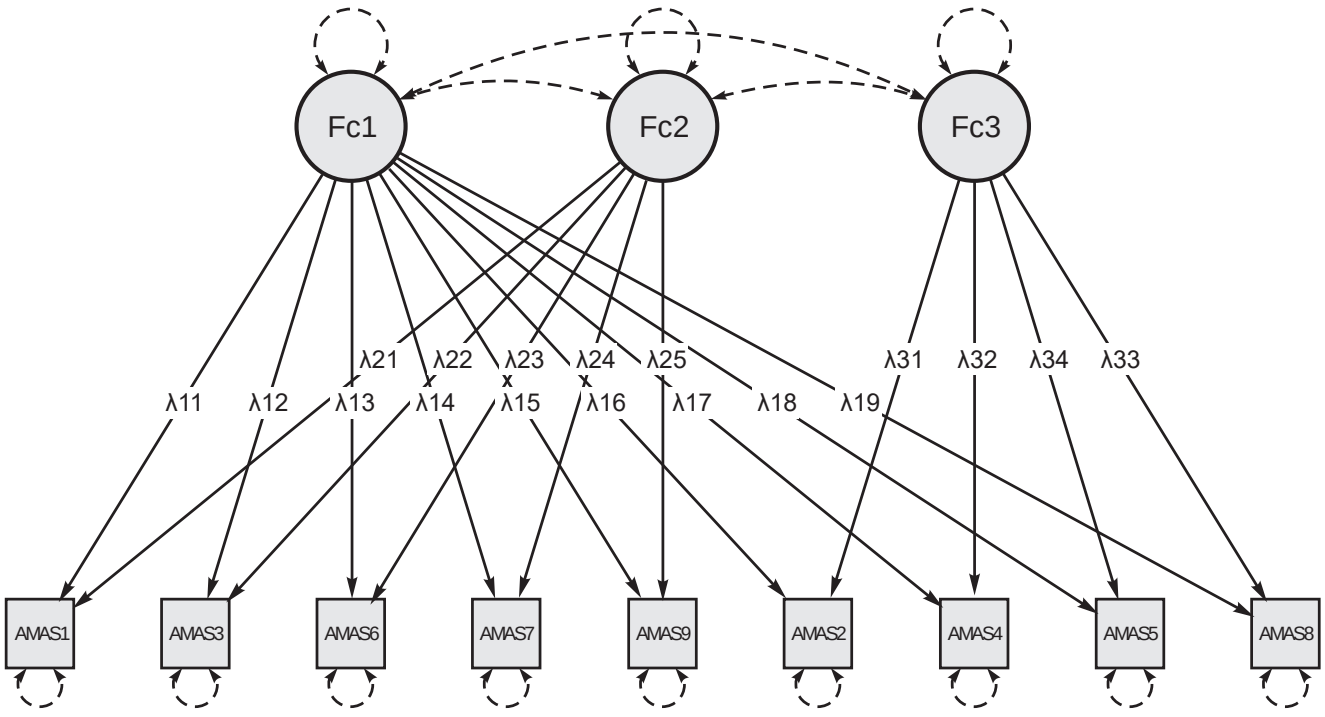


**FIG 2.**

*Model plot for factor structure of AMAS*

ty scales. The most common tool for assessing school anxiety in Russia is the Phillips School Anxiety Scale. However, it was adapted either in the form of a teacher's report, which does not allow the results to be directly correlated with the AMAS, which uses self-report, or for younger participants (grades 3–8) – our adaptation of the AMAS was carried out on schoolchildren in grades 10–11 [25–27]. The scope of this scale includes the manifestation of anxiety in educational activities, social stress at school, social behavior, which is not directly related to MA. This scale is quite difficult for screening because it consists of 8 subscales, including 58 questions. It is criticized in the literature [28]. These limitations do not allow the use of school anxiety scales to assess the external validity of the AMAS.

In our study, the external validity of the AMAS general scale was assessed by Spearman correlations with the STAI-T and Perceived Difficulty of Math. The STAI-T technique is highly reliable and is used in a variety of practices. It has proven itself well in previous studies assessing the external validity of the MA technique, in particular, in the recent study by M.I. Núñez-Peña and G. Guilera on the validation of the brief MA scale in a Spanish-speaking sample [29]. The STAI-T is also used to differentiate between different forms of anxiety. For example, in a study by L. Buratta et al. [30], the use of the STAI was able to differentiate the role of MA from other forms of anxiety and found that levels of trait anxiety and situational anxiety did not correlate with or negatively affect math performance. The use of the "Perceived Difficulty of Math" scale from the "Gender stereotypes and incremental beliefs about STEM" questionnaire allows, firstly, to additionally monitor the occurrence of a negative emotional state associated with mathematics (the item "Studying math gives me anxiety"), similar to the MA questionnaire SIMA, which consists of one question and has high correlations with the AMAS [31]. Secondly, the use of the scale provides to assess the student's self-esteem of abilities specifically in mathematics, and not within the framework of the entire educational process.

As a result, significant correlations between the AMAS and the used scales were found (Table 5).

**TABLE 5**

**CORRELATIONS BETWEEN AMAS TOTAL SCALE, STAI-T AND PERCEIVED DIFFICULTY OF MATH**

| Indices | AMAS Total |
|---|---|
| STAI-T | 0.38* |
| Perceived Difficulty of Math | 0.35* |

**Note.** * – $p < 0.001$.

### Gender differences and measurement invariance across genders

The next step in our analysis was to assess sex differences. The results are presented in Table 6. Overall, girls showed higher MA, which is consistent with previous studies. However, for LMA differences are insignificant.

Measurement invariance across genders was also investigated in this study (Table 7). Two models were tested: model 1 corresponds to configural invariance, model 2 characterizes metric invariance. The following indices were selected as demonstrating good model 1 fit: the CFI of > 0.95, and RMSEA < 0.08. CFI = 1.000 and RMSEA = 0.000 obtained for model 1 are within acceptable values, which demonstrates that the data is invariant in terms of configural invariance. Metric invariance (model 2) demonstrated ΔCFI = 0.000 and ΔRMSEA = 0.000, $p = 0.497$. As reported, ΔCFI < 0.010 and ΔRMSEA < 0.015 demonstrates metric invariance [32].

**TABLE 6**

**GENDER DIFFERENCES AND DESCRIPTIVE STATISTICS FOR LMA, MEA AND AMAS_TOTAL**

| Scales | Male | | Female | | Mean difference | *p*-value |
|---|---|---|---|---|---|---|
| | M | Sd | M | Sd | | |
| LMA | 1.8 | 3.2 | 2.2 | 3.4 | 0.5 | 0.02 |
| MEA | 5.6 | 3.8 | 7.6 | 3.9 | 2.0 | < 0.001 |
| AMAS_total | 7.4 | 6.0 | 9.9 | 6.4 | 2.5 | < 0.001 |

**TABLE 7**

**FIT INDICES FOR MEASUREMENT INVARIANCE ACROSS GENDERS**

| Models | CFI | RMSEA | Baseline test | | | Difference test | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\chi^2$ | Df | P | ΔCFI | ΔRMSEA | $\Delta\chi^2$ | Δdf | *p* |
| Model 1 | 1.000 | 0.000 | 31.749 | 36 | 0.671 | – | – | – | – | – |
| Model 2 | 1.000 | 0.000 | 46.284 | 51 | 0.661 | 0.000 | 0.000 | 14.373 | 15 | 0.497 |

## DISCUSSION

This study had several objectives, including: assessing the factorial validity of the AMAS questionnaire, assessing the internal consistency of the methodology, assessing the external validity of the scale, and analyzing sex differences and measurement invariance. All stated goals were achieved and the results are discussed in more detail below.

First, analysis of factor validity was carried out. A confirmatory factor analysis procedure was used to assess the factorial validity of AMAS. Three models were evaluated: one-factor, two-factor and bifactor (with a general MA factor and two subscales). The analysis showed that the bifactor model provided a better fit to the data. The obtained result is consistent with the conclusions of other studies demonstrating the best fit of this model [16, 33]. Thus, our version of the questionnaire consists of two subscales: LMA (5 items) and MEA (4 items). The distribution of questions on scales was the same as in previous studies [15]. This analysis allows us to measure math anxiety using AMAS both in a learning situation and in a situation of knowledge assessment.

Second, internal consistency was evaluated. In our study, high scores were obtained on the Cronbach's alpha for general math anxiety, as well as on the LMA and MEA subscales. This indicates a high internal consistency of the questionnaire. The results obtained are consistent with original version [15].

Third, external validity was estimated. A study of Cipora and colleagues has shown that mathematics anxiety correlates with general anxiety [17]; therefore, the Spielberger State – Trait Anxiety Inventory STAI-T was chosen to assess external validity. This study has shown low but significant correlations between AMAS and STAI-T [17], a similar result was observed in our study. We also used the Perceived Difficulty of Math questionnaire (scale of Questionnaire "Gender stereotypes and incremental beliefs about STEM") for the first time to test external validity. The results showed low but significant correlations. This may indicate that perceived difficulty of math and math anxiety are related constructs: math-anxious individuals perceive math as a complicated subject to tackle and become anxious when dealing with it. This can also work in reverse: anxiety prevents concentrating on math problem, so it is perceived by the student as difficult. Possible mechanism may be related to overload of working memory, which is consistent with the Deficient Inhibition Mechanism theory [8].

Finally, sex differences and measurement invariance across sexes were evaluated. The assessment of sex differences in AMAS in our study was provided separately on the LMA and MEA subscales and the total scale of math anxiety. This seems to be justified, since, on the one hand, studies describe an increase in math anxiety scores in girls, on the other hand, some report an increase in math anxiety only in the assessment situation. Therefore, it is important to separate these constructs in the analysis. Our results are consistent with previous studies [8]. An increase in general math anxiety, MEA, which characterizes specific anxiety in the assessment of math knowledge, and LMA, math anxiety in a learning situation were shown. Thus, older adolescent girls experience greater anxiety when studying and assessing their knowledge, which may be the result of the influence of gender stereotypes. Most likely, this increase in anxiety is due to the girls of this age being more exposed to gender stereotypes over their lifetime. The performed analysis of measurement invariance demonstrated configural invariance, which suggests the unity of the math anxiety construct measured by AMAS in both sexes. Also, our data demonstrated the similarity of factor loadings in boys and girls, as evidenced by metric invariance.

The strengths and weaknesses of the present study should be noted. One obvious advantage of this study is the sufficient sample size, which ensured the reliability of the statistical analysis and the conclusions drawn from it. Another advantage is that for the first time in the Russian-speaking sample, the invariance of the measurement by sex was assessed. As for limitations of this study, there is a lack of analysis of test-retest reliability, which should be carried out in future studies.

## CONCLUSIONS

AMAS demonstrated acceptable psychometric properties and factorial validity, which makes it a sufficiently effective tool to assess mathematics anxiety in high school students. The unique advantage of the scale lies in the possibility of distinguishing between the measurement of test anxiety in the situation of evaluating mathematical concepts, and mathematics anxiety in the situation of everyday learning. This scale can be useful in pedagogy. It can be used by practicing psychologists, for example, at school, to detect and prevent cases of mathematics anxiety in a timely manner, which can reduce the distress that occurs in the classroom. In general, this can make education more effective, as it assesses individual differences in anxiety and takes them into account when organizing the educational environment.

**Conflict of interests**

The authors of this article declare no conflict of interest.

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# IDEAS ABOUT THE STRESSFULNESS OF THE PARENTAL ROLE IN POTENTIAL PARENTS: OBJECTIVE AND SUBJECTIVE DETERMINANTS

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## ABSTRACT

*Studying stress factors of childbirth intentions is an important parameter in population development policy making. There is no data on the comparison of the relative importance of different types of determinants and stress inducing factors of parenting in the scientific literature.*

***The aim of the study.*** *To study the factors determining the ideas about the stressfulness of the parental role among modern youth.*

***Materials and methods.*** *The sample consisted of 364 students with no parenting experience. Three groups were identified, differing in their orientation towards having children: a group with a focus on single-child parenting (257 people); group with childfree focus (32 people); group with a focus on multi-child parenting (75 people). The following methods were used: socio-demographic questionnaire; "Parental Stress Scale" (J.O. Berry, W. Jones, 1995; adapted by Yu.V. Misiyuk, I.V. Tikhonova, 2022); "Intensive Parenting Attitudes Questionnaire" (M. Liss, H.H. Schiffrin, V.H. Mackintosh, H. Miles-McLean, M.J. Erchull, 2013; adapted by Yu.V. Misiyuk, 2022); express version of the "World Assumptions Scale" (R. Yanoff-Bulman; modified by M.A. Padun, A.V. Kotelnikova; author's semi-structured interview.*

***Results.*** *We analyzed the ideas about the stress that is associated with fulfilling the parental role. Parameters that potentially act as the factors determining the stressfulness of parenthood are considered. The specificity of ideas on parental stress and its determinants is analyzed in accordance with the dominant orientation towards childbearing.*

***Conclusion.*** *Ideas about the stressfulness of the parental role are determined by objective (age, status of personal relationships, education) and subjective (assessment of family relationship, attitudes towards the need to stimulate the child's development, essentialism and satisfaction from upbringing children) factors, but subjective ones have the greatest weight. The specificity of the determination of ideas about parental stress depending on the orientation towards childbearing has been revealed.*

***Key words:*** *parenting, ideas about parenting, parental stress, intensive parenting, stressful parenting role, youth*

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# ПРЕДСТАВЛЕНИЯ О СТРЕССОГЕННОСТИ РОДИТЕЛЬСКОЙ РОЛИ У ПОТЕНЦИАЛЬНЫХ РОДИТЕЛЕЙ: ОБЪЕКТИВНЫЕ И СУБЪЕКТИВНЫЕ ДЕТЕРМИНАНТЫ

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## РЕЗЮМЕ

*Изучение стресс-факторов намерений рождаемости является важным параметром формирования политики развития населения. В научной литературе отсутствует сравнение относительной важности различных видов детерминант и факторов стрессогенности родительской роли.*

***Цель исследования.*** *Изучение факторов, детерминирующих представления о стрессогенности родительской роли у современной молодёжи.*

***Методы.*** *Выборка состояла из 364 студентов без опыта родительства. Выделены три группы, различающихся по ориентации на рождениеь детей: группа потенциально малодетных родителей (257 человек); группа с чайлд-фри ориентацией (32 человека); группа с ориентацией на многодетность (75 человек). Использовались следующие методики: социально-демографическая анкета; «Шкала родительского стресса» (Parental Stress Scale; J.O. Berry, W. Jones, 1995; адаптация Ю.В. Мисиюк, И.В. Тихоновой, 2022); «Опросник установок на интенсивное родительство» (Intensive Parenting Attitudes Questionnaire; M. Liss, H.H. Schiffrin, V.H. Mackintosh, H. Miles-McLean, M.J. Erchull, 2013; адаптация Ю.В. Мисиюк, 2022); экспресс-вариант «Шкалы базисных убеждений» (World Assumptions Scale (WAS); R. Janoff-Bulman; модификация М.А. Падун, А.В. Котельниковой); авторское полуструктурированное интервью.*

***Результаты.*** *Нами были проанализированы представления о стрессе, который сопряжён с исполнением родительской роли. Рассмотрены параметры, потенциально выступающие как факторы, определяющие стрессогенность родительства. Проанализирована специфика представлений о родительском стрессе и его детерминант в соответствии с доминирующей ориентацией на деторождение.*

***Заключение.*** *Представления о стрессогенности родительской роли определяются объективными (возраст, статус личных отношений, образование) и субъективными (оценка семейных отношений, установки на необходимость стимуляции развития ребёнка, эссенциализм и получение удовлетворения от воспитания детей) факторами, однако наибольший вес имеют субъективные. Выявлена специфика детерминации представлений о родительском стрессе в зависимости от ориентации на деторождение.*

***Ключевые слова:*** *родительство, представления о родительстве, родительский стресс, интенсивное родительство, стрессогенность родительской роли, молодёжь*

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## INTRODUCTION

«Parenting as the main purpose of life», «happiness of motherhood/fatherhood» – all these clichés have turned into social stereotypes, which, however, have little relation to reality. Parenting as a source of stress and a cause of maladaptive mental states is increasingly considered by Russian and foreign science [1, 2]. Costs, inconveniences both material and psychological can aggravate the current situation in the life of parents [3]. The consequences of parental stress (PS) are wide-ranging and diverse: it is associated with poor health, poor marital relationships and child abuse, and interference with children's normal mental development [4]. It has been evidenced that perceived parental stress related to parental demands upon themselves can have a negative impact on maternal mental health and well-being [5], contribute to the dysfunctional development of parent-child and partner relationships, and thus represent risk factors for psychopathology of all members of the family system [6]. Consequently, examining stressors of parenting image is an important part of early identification of risks and targets for intervention to prevent more serious adverse outcomes.

The factors contributing to the stressogenicity of the parental role have now been systematised and identified into several groups [7]:

Socio-demographic indicators: gender (parental role is more stressful for mothers than for fathers) [1, 8]; age (young parents are more stressed, as well as parents over 37 years old) [9]; material security (indicated as the main factor of stressfulness of the paternal role) [10].

Social-psychological indicators: social support (insignificant support from family and society is associated with more negative emotions and more stress from upbringing) [1, 11]; presence of a conflict situation in the family [9].

Psychological characteristics of the child: infant temperament [12]; developmental disorders and childhood illnesses, behavioural problems of children [13], etc.

Psychological characteristics of the parent: difficulties in time allocation and differentiating the spheres of family and work [14]; physiological factors and sleep deficit [11], parenting style [3, 13]; parental competence and emotional state [15].

An important point to note, however, is that there is no comparison in the literature of the relative importance of different types of parental role stressors, which represent a key component of fertility orientation that should help optimise fertility promotion policies. Existing studies have focused on actual parents rather than young people who are potential parents and therefore key targets of fertility policy interventions. In doing so, it is crucial to understand what factors determine the stressfulness of parenting images among young people and, consequently, may influence attitudes towards childbearing intentions.

Analyses of the age of decision-making with regard to deliberate childlessness show that most child-free adults declare their intentions to remain childless early – in adolescence or, predominantly, in early childbearing (adolescence), while the age of decision-making does not show differences by gender subgroups [16]. Parenting attitudes are formed as relatively stable by adolescence, undergoing later only some changes under the influence of the life situation and other factors, and by adolescence, on the basis of the formed attitudes, an image-constructed content has already been formed, including individual and general cognitive schemes of parental actions in particular and parental behaviour in general at three levels: social, microsocial, personal and semantic. Accordingly, the perceptions of the difficulties associated with the fulfilment of the parental role, as well as one's own reproductive plans, which are expressed in the orientation to parenting that we are studying, make sense to study in adolescence [17, 18].

According to A. Eisen's theoretical scheme, the decision-making process about having a child is limited by certain "life factors" (i.e. subjective and contextual conditions) [19]. Childbearing intentions are associated with individual socioeconomic status, including family income, human capital (i.e. education/health) and quality of employment, which is conventionally categorised in the literature as objective factors. Studies are available examining perceptions of future parenting in relation to factors such as gender and age [17]. Adolescence is ascertained as a period of formation of more adequate and definite ideas about parenting; gender specificity is also revealed, which is manifested in the advanced nature of formation of ideas about future parenting in girls. A subjective factor such as perception of parental role is also associated with heterogeneity in fertility intentions [20]. The system of value orientations acts as a condition for the formation of a harmonious attitude to parenting, and a high value of parenting is associated with a positive attitude to motherhood and fatherhood and to oneself as a parent. Peculiarities of parental families [21], self-awareness and conscious regulation [22] are also considered as factors influencing the formation of perceptions of motherhood and fatherhood. S.V. Merzlyakova et al. performed a systematic study of macro-, meso- and micro-level factors that determine different components of family self-determination. The role of such factors as gender, age, cognitive abilities, temperament properties, emotional and volitional features of personality, competence in communication, responsibility, moral orientations is demonstrated at the micro level [23].

Thus, a large volume of literature has been published that has theoretically or empirically discussed fertility intention and its determinants. Numerous situational, socio-economic, child and parental factors contributing to PS, quite widely studied in domestic and foreign literature, are not exhaustive characteristics that influence the stressoriness of the parental role image in potential parents. At the same time, in-depth knowledge about the relative importance of various factors influencing the subjective perception of parenting and specifically the image of parental role stressors is still lacking. However, it is crucial to reveal them in order to be able to help reduce stress during the childbearing planning stage. The significant

role of subjective psychological factors of the image of parenting stressogenicity remains beyond the scope of the study, forming the problem field of the present study. Moreover, the study of constraints (stressors) affecting fertility intentions is an important basis for shaping population development policies.

As a result, **the purpose of the study** was determined by the exploratory study of the factors determining the perceptions of stressfulness of the parental role in modern youth. In accordance with the theoretical framework, the current study considers personal and contextual determinants of the perception of parental role stressoriness, and they are subdivided into two conventional measurement vectors: objective (socio-demographic and anamnestic characteristics, gender, age, education, marital status, type of parental family, socio-economic status) and subjective (basic beliefs of the individual, attitudes about the intensity of parenting, assessment of their own child-parent and family relationships).

The choice of the mentioned subjective factors is oriented on modern research of stress mechanisms and factors, among which a significant role is assigned to beliefs and attitudes. Baseline beliefs are defined as «an individual's implicit global, stable beliefs about the world and himself/herself that influence the thinking, emotional state and behavior of a person» [24] as one of the significant mechanisms for the formation of intense stress and posttraumatic recovery. Their influence over essential aspects of a person's social life, interaction and relationships with surrounding people has been demonstrated [25]. Among the dominant trends and approaches to upbringing is a variation of conscious, super-inclusive parenting, which implies excessive involvement of modern men and women in the development of the child, parental determinism and comprehensive development of the child to achieve a higher social status. This approach in the Western and domestic scientific discourse is represented by the attitudes to «intensive parenting» [26]. Intensive parenting attitudes can act as a stressor of the parental role, detrimentally affecting the mental health of parents [3], associated with decreased life satisfaction, a constant sense of burden due to the parental role, and reflecting the severity of the demands of parenting, which in the context of increased expectations of the parenting role from society forms a stable subjective attitude to the perception of fatherhood and motherhood long before the appearance of children.

The main ***hypothesis of the study*** is the assumption of the predominant influence of subjective factors over the perceived stressfulness of parental role representations.

## METHODS

To investigate the perceptions of stressfulness of the parental role among potential parents, the Parental Stress Scale (Parental Stress Scale; J.O. Berry, W. Jones (1995), Russian version adapted from Y.V. Misiyuk and I.V. Tikhonova (2022)) was used, which reveals the general level of PS (in our study – perceptions of PS level) regardless of socio-demographic and situational indicators, and also allows to determine the expression of PS parameters (parental stressors, loss of control, parental reward/damage, parental satisfaction/dissatisfaction) [27].

A socio-demographic questionnaire was used to observe objective factors, and the following techniques were applied to study subjective factors:

- the socio-psychological section of the questionnaire, which surveyed the assessment of relationships in the family, orientations towards marriage, family relations, and childbearing;
- Intensive Parenting Attitudes Questionnaire (M. Liss, H.H. Schiffrin, V.H. Mackintosh, H. Miles-McLean, M.J. Erchull (2013); adaptation by Y.V. Misiyuk (2022)), which diagnoses the expression of attitudes towards intensive parenting, which include essentialism, satisfaction, difficulty, stimulation, and detocentrism [26];
- express variant of the World Assumptions Scale (WAS) questionnaire (R. Janoff-Bulman; modified by M.A. Padun and A.V. Kotelnikova). The original form of the questionnaire was modified. From each scale proposed by the authors («Self-image», «Benevolence of the surrounding world», «Justice», «Luck», «Beliefs about control»), the statement that has the highest factor loadings according to the results of psychometric testing was selected [25]. These statements were offered to respondents in the form of visual scales, the degree of agreement with the statement was assessed in percentage expression on the scale.

In the text, results are presented in the format of mean scores and percentage expression, taking into account the maximum possible values (parameters are given for each indicator). Percentage scores should be used when assessing scale severity and for ease of comparison of results.

The instructions for the questionnaires were subject to modification; respondents were asked to give answers in terms of perceptions of a hypothetical relationship with a future child.

The following statistical procedures of the SPSS Statistics 22 software package (IBM Corp., USA) were used: descriptive statistics (for an overview description of the results of the research instruments); Spearman's correlation coefficient (to determine correlations between variables); regression analysis (stepwise ridge regression method – to identify the influence of variables).

The study was based on the principle of voluntariness; all respondents gave informed consent to participate. Interviewees were informed of the purpose of the study and their rights. The principles of confidentiality and environmental friendliness were respected. The study was approved by the local ethical committee of the Kostroma State University (Minutes No. 2 dated April 22, 2022).

## RESULTS

The sample (generalised characteristics are summarised in Table 1) consisted of 364 childless students (218 (59.9 %) girls, 146 (40.1 %) boys) who receive pedagogical, medical, sociological, engineering and technical education in higher educational institutions of Kostroma and Yaroslavl. The mean age was 19.43 years (SD = 1.87).

In the sample there is a significant representation (26.9 %) of the group of respondents with «incomplete higher» education – these are persons who have not completed higher education in the past or who have changed their speciality of education; 47.53 per cent of young people are not in a relationship. 71.15 per cent were brought up in a small family, 19.5 per cent of respondents had no siblings.

The results of descriptive statistics (Table 2) demonstrate that the overall level of PS in the perceptions of young people corresponds to moderate values. Parental dissatisfaction is the least expressed in the PS «profile»; increased level of demands to the parent («stressors»), the probability of losing control over one's own life and the deficit of emotional rewards from the realisation of the parental role are recognised.

Prospective parents perceive the parenting role as «intense». They have beliefs about the need to actively stimulate children's development, an attitude of perceiving the parental role as time-consuming and perceive the child as the centre of attention in the family. To a lesser extent, the attitude to essentialism is expressed, i.e. the respondents are not oriented to the mother's primacy in child care and upbringing.

Generally, young people show a prevalence of positive basic beliefs about the «Self-image», demonstrate stable perceptions of control over their own lives and events, but to a lesser extent believe in their own luck, fairness and benevolence of the world around them. Child – parent and family relationships are rated quite highly by respondents (M = 8.19 and M = 8.14 out of 10).

Let us present a scheme reflecting the dependence of parental stress parameters over objective and subjective characteristics (Fig. 1).

The overall regression was statistically significant for measures of total expected PS and its components. It was revealed that they were significantly predicted by objective factors such as age, personal relationship status and education, and subjective factors such as evaluation of family relationships, attitudes towards the need to stimulate child development, essentialism and gaining satisfaction from child upbringing. Overall PS ($R$ = 0.61; $R^2$ = 0.37; $p$ = 0.000) was positively predicted by attitudes towards intensive parenting: difficulty (β = 0.13; $p$ = 0.004) and stimulation (β = 0.14; $p$ = 0.001) – as well as respondent age (β = 0.13; $p$ = 0.004); while it was negatively determined by family relationship evaluation (β = -0.13; $p$ = 0.003).

The assessment of the parental role as stressful (level of «parental stressors») ($R$ = 0.66; $R^2$ = 0.44; $p$ = 0.000) was positively contributed by the absence of a partner (single/unmarried status) (β = 0.08; $p$ = 0.05), level of education (incomplete higher education) (β = 0.11; $p$ = 0.01), essentialism attitude (β = 0.10; $p$ = 0.01); negative contribution – subjective evaluation of family relations (β = -0.15; $p$ = 0.0006).

Perceptions of potential limitations and emotional discomfort from the parental role («parental damage») are statistically significantly positively predicted ($R$ = 0.45; $R^2$ = 0.20; $p$ = 0.000) by respondent age (β = 0.13; $p$ = 0.008), stimulation attitude (β = 0.19; $p$ = 0.00006), and negatively by parental role satisfaction attitude (β = -0.19; $p$ = 0.0002).

**TABLE 1**

**DESCRIPTIVE STATISTICS OF OBJECTIVE DETERMINANTS/FACTORS IN THE YOUTH SAMPLE ($n$ = 364)**

| Sex ($n$) | | Age (M) | | Education ($n$) | | | |
|---|---|---|---|---|---|---|---|
| male | female | male | female | mean | vocational secondary | incomplete higher | higher |
| 146 | 218 | 19.43 | 19.37 | 184 | 58 | 98 | 24 |
| Marital status ($n$) | | | | Presence of siblings ($n$) | | | |
| single/ unmarried | romantically involved | legally married | | 1 | 2 | 3 or more | no |
| 179 | 173 | 12 | | 188 | 73 | 32 | 71 |
| Parental family characteristics ($n$) | | | | Type of activity ($n$) | | | |
| traditional family | | single-parent family/ divorced | | students | | employed students | |
| 261 | | 96 | | 364 | | 68 | |

**TABLE 2**

**DESCRIPTIVE STATISTICS OF SEVERITY INDICES OF PERCEPTIONS OF PARENTAL ROLE STRESSORS AND PERCEIVED SUBJECTIVE DETERMINANTS/FACTORS IN THE YOUTH SAMPLE (*n* = 364)**

| | Parental stress parameters | | | | |
|---|---|---|---|---|---|
| | Parental stressors (max – 30) | Parental reward/ damage (max – 25) | Loss of control (max – 20) | Parental satisfaction/ dissatisfaction (max – 15) | Overall parental stress level (max - 90) |
| M (%) | 15.37/51.22 | 12.11/48.45 | 9.11/45.55 | 6.63/36.86 | 44.37/49.30 |
| σ | 4.34 | 6.36 | 3.29 | 2.85 | 12.78 |
| | Parameters of attitudes towards intensive parenting | | | | |
| | Essentialism (max – 48) | Satisfaction (max – 24) | Stimulation (max – 24) | Difficulties (max – 36) | Child centrism (max – 18) |
| M (%) | 22.48/46.84 | 17.07/71.14 | 18.74/72.15 | 26.75/74.31 | 11.37/63.19 |
| σ | 5.62 | 3.53 | 3.11 | 3.50 | 2.43 |

| | Parameters of baseline beliefs and assessment of child-parent family relationships | | | | | | |
|---|---|---|---|---|---|---|---|
| | Self-image (max - 100) | Benevolence of the surrounding world (max – 100) | Justice (max – 100) | Luck (max – 100) | Control conviction (max - 100) | Assessment of child-parent relationships (max - 10) | Assessment of family relations (max - 10) |
| M | 77.13 | 52.47 | 62.32 | 61.41 | 71.07 | 8.19 | 8.14 |
| σ | 47.86 | 24.58 | 26.91 | 22.55 | 21.28 | 1.91 | 2.03 |

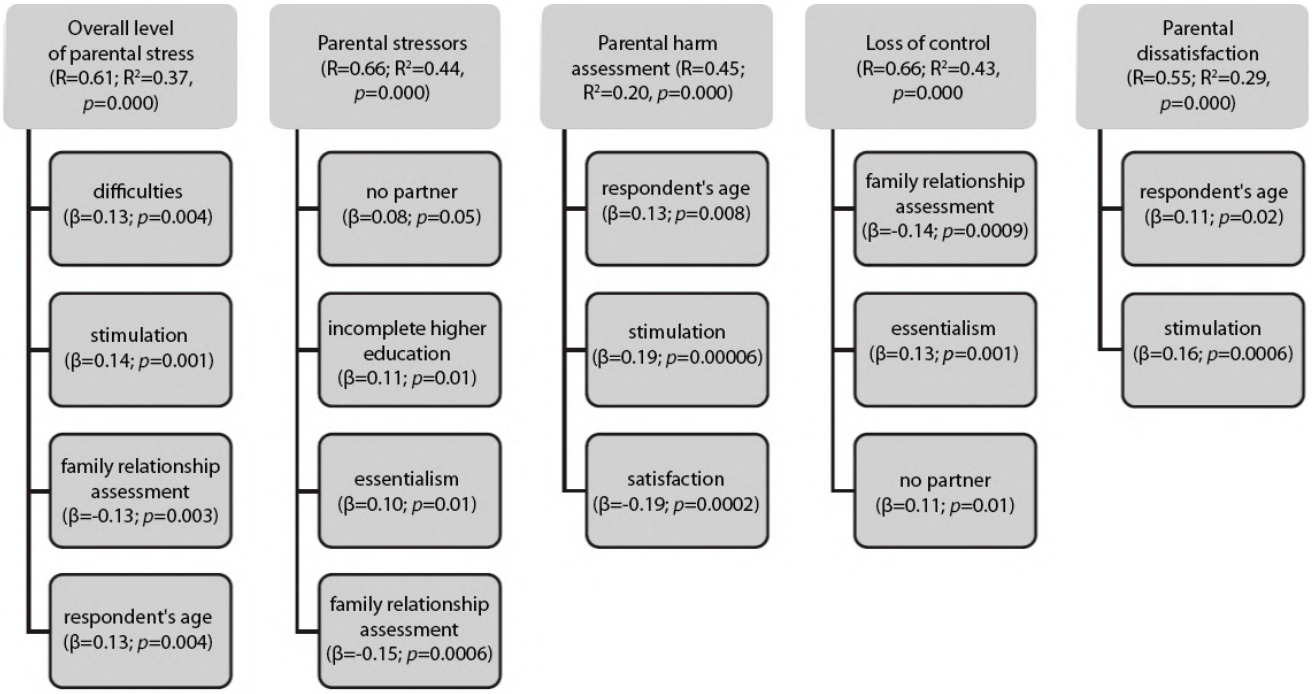



***FIG. 1.***
*Regression analysis of factors predicting perceptions of parental stress among youths*

The expectation of losing control over their own lives among potential parents is positively determined ($R = 0.66$; $R^2 = 0.43$; $p = 0.000$) by the fact of not having a partner ($\beta = 0.11$; $p = 0.01$), by the attitude of essentialism ($\beta = 0.13$; $p = 0.001$), and negatively by the evaluation of family relationships ($\beta = -0.14$; $p = 0.0009$).

The important PS indicator «parental dissatisfaction» in young people's perceptions will be positively predicted ($R = 0.55$; $R^2 = 0.29$; $p = 0.000$) by respondent age ($\beta = 0.11$; $p = 0.02$) and attitudinal stimulation ($\beta = 0.16$; $p = 0.0006$).

The sample was divided into three empirical groups by childbearing orientation and based on the respondents' answers in the questionnaire about the desired number of children in the future. Within the study group, the following were distinguished: a group of potentially childless parents with an orientation towards the classical family structure with 1–2 children ($n = 257$; M = 19.45; SD = 1.86); a group of child-free parents who declared their unwillingness to have children in the future ($n = 32$; M = 18.78; SD = 1.48); a group of persons with an orientation towards having many children ($n = 75$; M = 19.45; SD = 2.01), who noted their intention to have 3 or more children (Table 3).

Among the socio-demographic characteristics in the identified empirical groups, the following should be highlighted as key features. Girls (61.9 %) are more oriented towards having few children than boys (38.1 %); the majority are in a romantic relationship (52.5 %) or in an official marriage (4.7 %); this group has the highest percentage of employed (21 %) young people who already have secondary vocational education (16.7 %); the majority grew up in a complete family (70.8 %); the highest percentage of young people without siblings (22.2 %) among all groups. There are more girls in the group with orientation on having many children (58.7 %); 50.7 % of respondents are single/unmarried, 45.3 % are in romantic relations/civil marriage; 17.3 % combine study and work; 16 % have secondary specialised education; the majority (74.7 %) are from a complete family; 12 % have no siblings, while 45.3 % have 2–4 siblings (respondents were brought up in a large family themselves). The group with childlessness orientation has more young men (53.1 %); the smallest percentage among the groups of working students (3.1 %) and those with education (specialised secondary education – 9.4 %); the majority (71.9 %) were brought up in a complete family; respondents mostly have no romantic relations (84.4 %); 15.6 % have no siblings.

The analysis has revealed that the level of parental role stressfulness statistically significantly differs in the perceptions of young people with different childbearing orientation. The maximum stressfulness of the parental role is determined in the perceptions of students talking about unwillingness to have children. They observed a high level of overall expression of parental stress ($n = 32$; M = 58.44 or 64.93 % of the maximum score). In the «profile» of its severity, a prevalence of indicators related to the demands and limitations of the parent: «Parental stressors» (M = 21.47 or 71.56 % of the maximum score) and «Loss of control» (M = 13.94 or 69.69 % of the maximum score) can be observed. Respondents whose preferred family form is a large family have the lowest parental stress scores. The perceptions of parenting among respondents who prefer many and few children are statistically significantly different in terms of «Parental Stressors» (M = 13.81 or 46.04 % of the maximum score; M = 15.00 or 50.19 % of maximum score respectively), «Loss of Control» (M = 7.85 or 39.26 % of maximum score; M = 8.88 or 44.37 % of maximum score respectively), «Parental Dissatisfaction/Satisfaction» (M = 5.84 or 32.44 % of maximum score; M = 6.60 or 36.10 % of maximum score respectively).

Correlations were determined to reveal factors associated with PS perceptions among youth with different childbearing orientations (Fig. 2, 4, 6), and regression analyses were conducted to reveal determinants predicting increased parental role stressors in young people's perceptions (Fig. 3, 5, 7).

In the group of persons with orientation towards having few children, among objective stressors we can observe the following anamnestic characteristics: age, female gender, absence of a partner (single person/unmarried girl position) (Fig. 2). In the group of persons with orientation towards having few children, among objective stressors we can observe the following anamnestic characteristics: age, female gender, absence of a partner (single person/unmarried girl position) (Fig. 2). Importantly, contextual «objective» factors have weak correlations with PS parameters compared to «subjective» factors, which have moderately strong correlations. Specifically, high significance level negative relationships of all indicators of perceived parental stress were revealed with satisfaction attitudes. Beliefs about the mother's leading role in child upbringing may be positively related to the stressor of parenting in young people's perceptions. The interaction of the loss of control parameter with the assessment of family and child-parental relations is also characterised by strong negative links (Fig. 2). A slightly different picture was obtained in analysing the causal relationships between parental stress symptoms and different groups of factors (Fig. 3).

It appeared that in the group of potentially young people with an orientation towards having few children, perceptions of the overall level of parental stress were determined by only one objective factor, the age of the respondent ($R = 0.20$; $R^2 = 0.42$; $p = 0.001$; $\beta = 0.19$). But its strengthening is predicted by a complex of subjective factors ($R = 0.48$; $R^2 = 0.23$; $p = 0.000$): the expression of attitudes about the mother's primary role in child upbringing ($\beta = 0.32$; $p = 0.00$), the need to stimulate the child's development ($\beta = 0.21$; $p = 0.00$), and the deficit of beliefs in the possibility of getting satisfaction from parenting ($\beta = -0.30$; $p = 0.00$). The remaining parameters of parental stress are influenced only by subjective factors,

among which the greatest role is played by attitudes towards the intensity of upbringing. Perceptions of high levels of parental role demands – «parental stressors» – will be reinforced ($R$ = 0.50; $R^2$ = 0.25; $p$ = 0.000) in the presence of low valuation of family relationships (β = -0.17; $p$ = 0.002), doubts about being satisfied with parenting (β = -0.35; $p$ = 0.000) and beliefs about its labour-intensiveness (β = 0.29; $p$ = 0.000). Perceptions of low reward from the realisation of parental functions: «parental damage» is determined ($R$ = 0.39; $R^2$ = 0.15; $p$ = 0.000) by a combination of the expression of attitudes of essentialism (β = 0.31; $p$ = 0.0000), stimulation (β = 0.25; $p$ = 0.00002), but low belief in the need to prioritise the needs of the child – child-centrism (β = -0.20; $p$ = 0.0005). Loss of control over one's own life as a symptom of parental stress was predicted ($R$ = 0.53; $R^2$ = 0.28; $p$ = 0.000) by a combination of essentialism attitudes (β = 0.18; $p$ = 0.0004), parental role difficulty (β = 0.19; $p$ = 0.0003) and satisfaction deficits (β = -0.35; $p$ = 0.000), low valuation of family relationships (β = -0.19; $p$ = 0.0005). Parental dissatisfaction as a PS trait was determined by the same set of attitudes concerning upbringing intensity as the overall PS level ($R$ = 0.43; $R^2$ = 0.19; $p$ = 0.000): essentialism (β = 0.26; $p$ = 0.000), stimulation (β = 0.20; $p$ = 0.0003), and beliefs about parental satisfaction deficits (β = -0.29; $p$ = 0.000).

**TABLE 3**

**DESCRIPTIVE STATISTICS OF SOCIO-DEMOGRAPHIC CHARACTERISTICS OF THE YOUTH SAMPLE IN THE EMPIRICAL GROUPS WITH DIFFERENT CHILDBEARING ORIENTATION (*n* = 364)**

| Characteristics | Orientation towards «with few children» (*n* = 257) | Orientation towards «having many children» (*n* = 75) | Orientation towards «childlessness» (*n* = 32) |
|---|---|---|---|
| Age (M) | 19.45 | 18.78 | 19.45 |
| **Sex, *n* (%)** | | | |
| Girls | 159 (61.9) | 44 (58.7) | 15 (46.9) |
| Young men | 98 (38.1) | 31 (41.3) | 17 (53.1) |
| **Type of activity, *n* (%)** | | | |
| Students | 236 (91.8) | 70 (93.3) | 31 (96.9) |
| Employed | 21 (8.2) | 5 (6.7) | 1 (3.1) |
| Employed students | 54 (21.0) | 13 (17.3) | 1 (3.1) |
| **Marital status, *n* (%)** | | | |
| Single/unmarried | 110 (42.8) | 38 (50.7) | 27 (84.4) |
| Romantically involved | 135 (52.5) | 34 (45.3) | 5 (15.6) |
| Legally married | 12 (4.7) | 3 (4.0) | 0 |
| **Education, *n* (%)** | | | |
| Mean | 131 (60.0) | 37 (49.3) | 17 (53.1) |
| Vocational secondary | 43 (16.7) | 12 (16.0) | 3 (9.4) |
| Incomplete higher | 70 (27.2) | 18 (24.0) | 10 (31.3) |
| Higher | 15 (5.8) | 8 (10.7) | 2 (6.2) |
| **Parental family characteristics, *n* (%)** | | | |
| Traditional family | 182 (70.8) | 56 (74.7) | 23 (71.9) |
| Single-parent family/divorced | 75 (29.2) | 19 (25.3) | 9 (28.1) |
| **Presence of siblings, *n* (%)** | | | |
| No (subject is an only child) | 57 (22.2) | 9 (12.0) | 5 (15.6) |
| 1 sibling (small family) | 134 (52.1) | 32 (42.7) | 23 (71.9) |
| 2-4 siblings (large family) | 66 (25.7) | 34 (45.3) | 4 (12.5) |

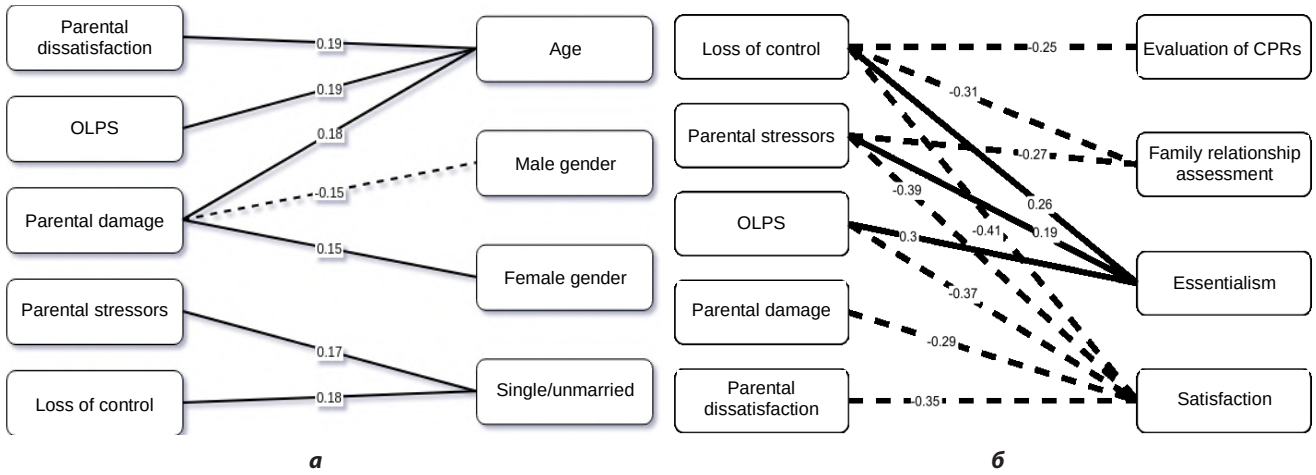



***FIG. 2.***
*Correlations of the level of perceived parental stress indices among young people with orientation to having few children:* ***а*** *– with objective characteristics;* ***б*** *– with subjective characteristics; solid line – direct correlations; dotted line – inverse correlations. Statistical significance level: medium thickness line – p ≤ 0.01; thick line – p ≤ 0.001*

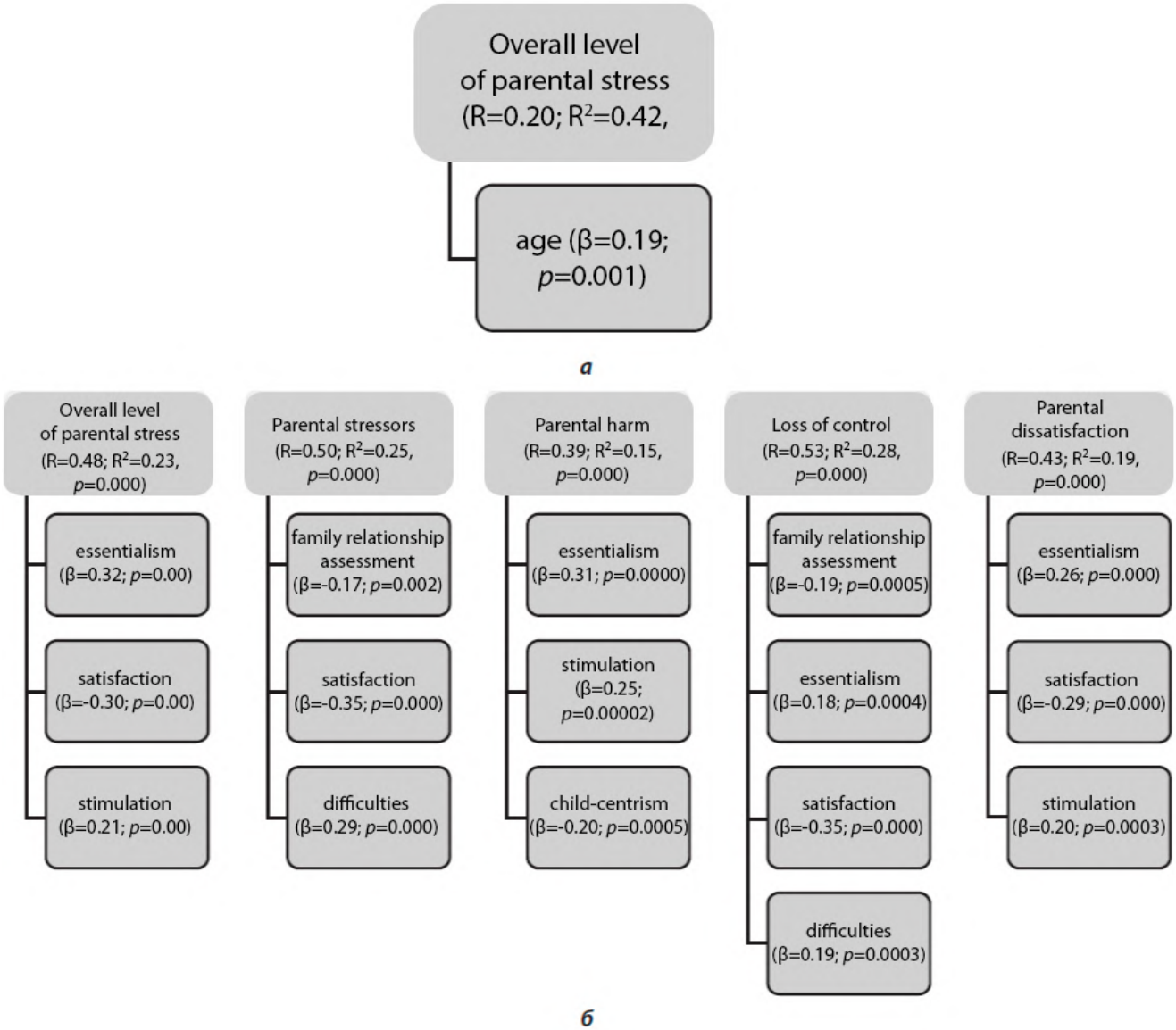



***FIG. 3.***
*Determination of anticipated parental stress level indicators among young people with an orientation towards having few children:* ***а*** *– objective characteristics;* ***б*** *– subjective characteristics*

The analysis of correlations in the group of respondents with child-free orientation reveals almost complete absence of correlations between stressor and anamnestic characteristics (Fig. 4), which provides us with prospects for studying child-free orientation from the point of view of its conditioning by one's own experience. It is worth paying attention to the influence of the position from the only child in the family onto the perceptions of stressor of parenting in the form of loss of control, as well as to the greater strength of correlations of the negative nature of subjective factors with all parameters of parental stress. Again the leaders in mutual influence are the attitudes to satisfaction (Fig. 4).

In the group of young people with a child-free orientation, the only determinant of parental stress symptoms is the attitude of being able to enjoy parenting («satisfaction»). It negatively predicted individual attributes rather than overall stress levels, namely parental stressors ($R$ = 0.51; $R^2$ = 0.26; $p$ = 0.003; β = -0.49), loss of control ($R$ = 0.52; $R^2$ = 0.27; $p$ = 0.003; β = -0.49) and parental dissatisfaction ($R$ = 0.58; $R^2$ = 0.34; $p$ = 0.0005; β = -0.55).

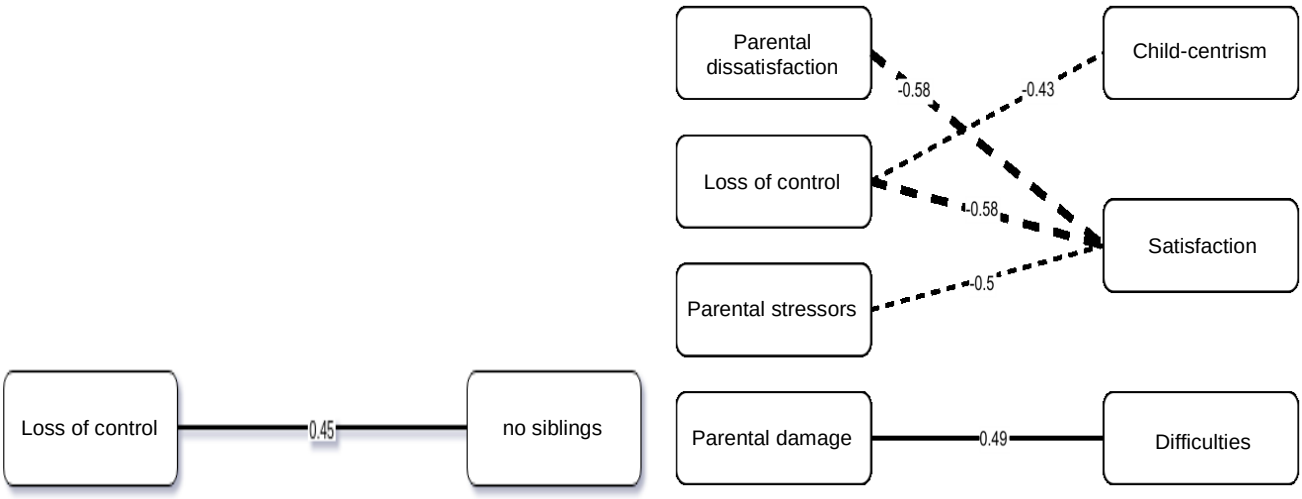
In the group of young people with orientation towards having many children, strong links of stressor indicators with female gender and, on the contrary, negative links with male gender are observed (Fig. 6).

Among personal subjective factors (Fig. 6), it is in this group that the basic beliefs about the positivity of self-image and one's ability to maintain control, as well as the assessment of child-parental relations also become related to the idea of stressor parenting.

The analysis of the determinacy of PS perceptions among young people with a multi-child orientation revealed the influence of only a single objective factor (level of education) over the parental stressors parameter ($R$ = 0.40; $R^2$ = 0.16; $p$ = 0.000; β = 0.39). In other words, having an incomplete higher education predicts increased perceptions of high levels in parental role demands. The influence of subjective factors alone was also determined in the remainder. The expression of overall parental stress levels ($R$ = 0.43; $R^2$ = 0.19; $p$ = 0.0001; β = 0.41), its symptoms in the form of «parental damage» ($R$ = 0.41; $R^2$ = 0.17; $p$ = 0.0002; β = 0.39) and parental dissatisfaction ($R$ = 0.42; $R^2$ = 0.18; $p$ = 0.0002; β = 0.40) are predicted by the expression of essentialism attitudes. Low levels of parenting pleasure attitudes predict PS «loss of control» symptom severity ($R$ = 0.39; $R^2$ = 0.15; $p$ = 0.0006; β = -0.37). The formation of perceptions of high demands of parenting – «parental stressors» – is being determined by a group of factors ($R$ = 0.56; $R^2$ = 0.32; $p$ = 0.0000) including attitudes of labour-intensiveness (β = 0.39; $p$ = 0.0001) and low satisfaction (β = -0.48; $p$ = 0.000004) with parenting.

## DISCUSSION

Analysing the determinants of perceptions of parental role stressor in a general sample of prospective parents. Among contextual objective determinants of perceptions of parental role stressor in the general youth sample, age makes a significant contribution to the predetermination of perceptions of stressor, which is consistent with available studies about the development of the system of parenting perceptions [17], and can also be explained by the expansion of social experience, cognitive development, and increased level of personal maturity. Apparently, these processes can also explain the role of the education factor. Perhaps higher education that enlightens about developmental patterns shapes the expectation of stressor, as it speaks mostly about crises and difficulties of different age periods and little about options for overcoming them.




*а* *б*

***FIG. 4.***
*Correlations of the level of perceived parental stress indices among young people with child-free orientation:* ***а*** *– with objective characteristics;* ***б*** *– with subjective characteristics; solid line – direct correlations; dotted line – inverse correlations. Statistical significance level: medium thickness line – p ≤ 0.01; thick line – p ≤ 0.001*

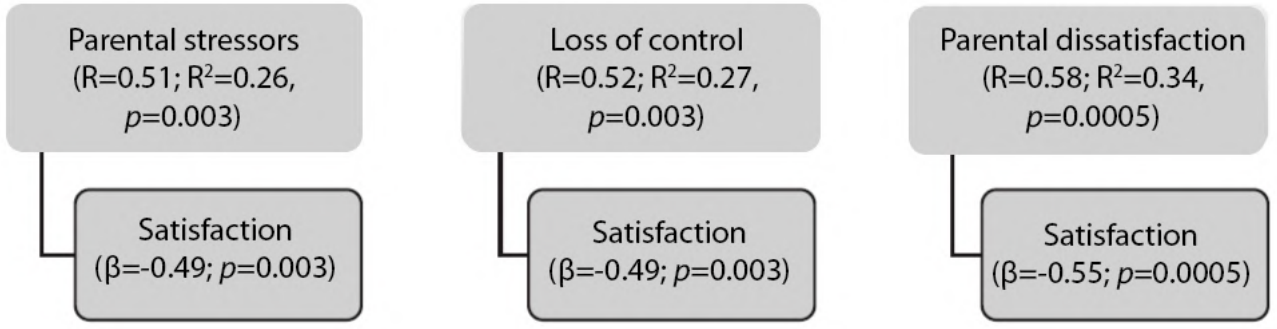



***FIG. 5.***
*Indices of perceived parental stress level in youth with childfree orientation determinants*

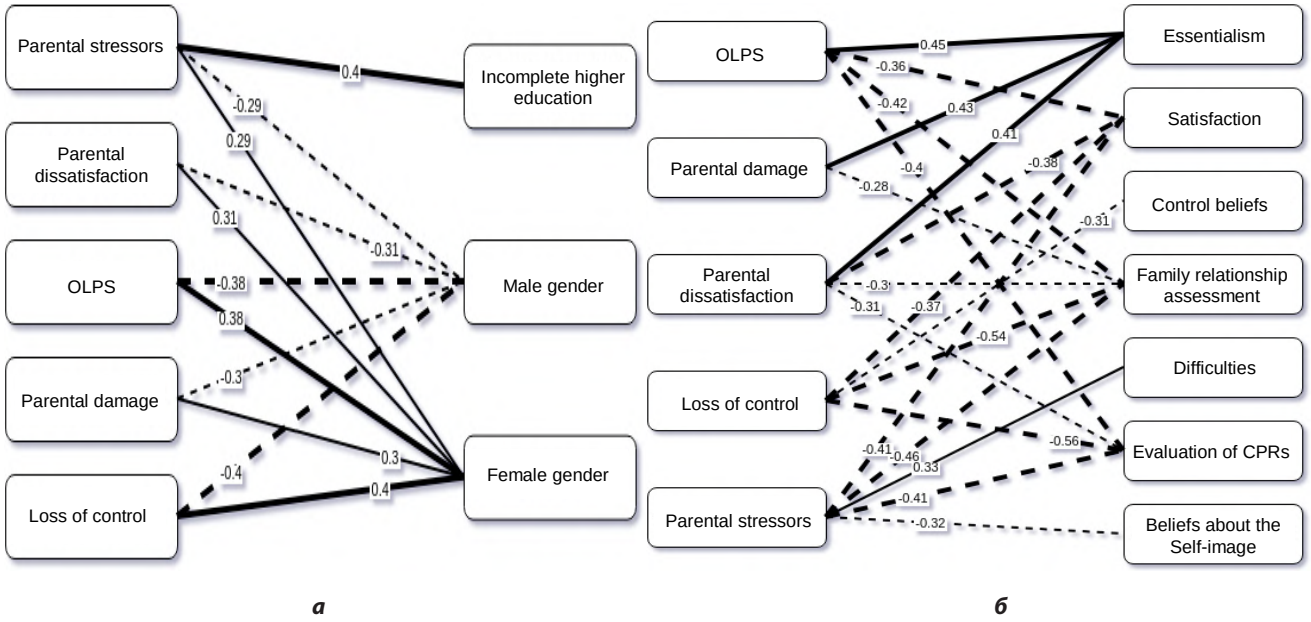



***FIG. 6.***
*Correlations of the level of perceived parental stress indices among young people with orientation to having many children:* ***а*** *– with objective characteristics;* ***б*** *– with subjective characteristics; solid line – direct correlations; dotted line – inverse correlations. Statistical significance level: medium thickness line – p ≤ 0.01; thick line – p ≤ 0.001*

The existing experience of failure in higher education (unfinished higher education) increases stressors in terms of perceptions of high demands to parents (parental stressors), which, presumably, may be related to the expectation of future uncertainty.

Highlighting «lack of partner» as a factor in hypothetical PS was not unexpected, as in general the single-parent family is considered more vulnerable and the parent is more susceptible to the difficulties associated with role overload. It is also consistent with the important role of social support in the occurrence of PS [12]. Some studies indirectly identify the factor of partner absence as a stressor because it is associated with lower parental role satisfaction [8].

Gender does not emerge as a determinant of parenting stressor in relation to perceptions of parental stress in the cumulative sample. It may however be a factor influencing these perceptions in separate analyses of data from a group of potentially small number of child parents. This seems to be following gender stereotypes (the idea that women play a greater role in child upbringing and, as a consequence, are more stressed). A number of studies have shown that mothers experience less satisfaction with parenting than fathers [1, 3]. In the context of the essential attitudes expression of intensive parenting, women often experience societal pressures to conform to cultural standards of highly participatory parenting [26]. Likewise, following L. Ruppanner et al. (2019), this result tends to be justified by the greater orientation of potentially mothers with few children towards a primary role in childcare and upbringing, as well as the predominant fulfilment of household duties [28]. It is worth noting that economic status, parental family type, divorce, and a number of other factors which have been suggested by this study were also not determinants of stressor perceptions of the parental role in the overall sample of potential parents.

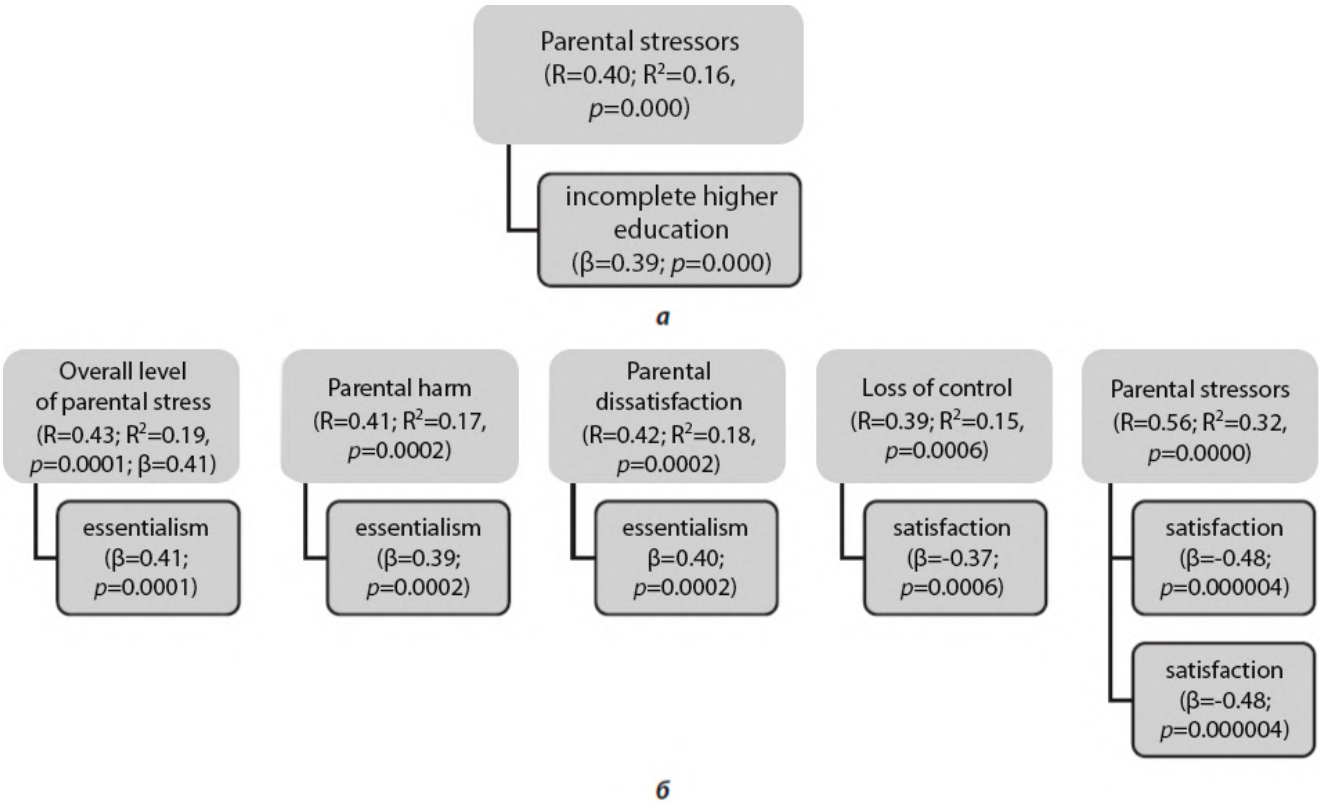



***FIG. 7.***
*Determination of anticipated parental stress level indicators among young people with multiple-childhood orientation:* ***а*** *– objective characteristics;* ***б*** *– subjective characteristics*

Among personal «subjective» micro-level factors that are determinants of parental role stressor in young people's perceptions, attitudes to upbringing intensity play a major role. An interesting fact is the established negative influence of the attitude of satisfaction with parenting in young people's perceptions of PS. This orientation is seen as a variant of self-actualisation, when active child upbringing can bring positive results and pleasure, respectively, can act as a factor reducing perceptions of parenting stressor. Similar findings were obtained previously in a sample of mothers whose stress was low in the presence of beliefs about the rewarding nature of parenting [29].

Attitudes about the need to stimulate child development from an early age are a factor that reinforces the perception of the parental role as stressful. This relationship is also observed in groups of young people oriented towards childlessness or having few children, emphasising its role in fertility plans. The identified pattern confirms the negative role of parental determinism as a total social attitude that creates orientations to «proper parenting» [30].

It is worth noting the large number of links of the dimensions of perceived parental stress with the perception of the parental role as difficult (parenting is perceived as a difficult life task, a «challenge», a «labour-intensive project») and essentialism beliefs (women are more competent parents and bear the main burden of care and upbringing). The highlighted subjective factors of parental role stressor in the youth environment are also characteristic for parents with children. Men and women who found parenting challenging were prone to depression and showed higher levels of stress, while experiencing lower life satisfaction. Women's belief that «motherhood is a challenge» reduces their sense of competence as a parent, leading to poorer well-being [29].

A significant contribution to the perceptions of the parental role stressor is made by the factor of evaluation of the respondents' family relationships. It should be noted that the assessment of child-parents' relationships did not become a determinant (although it is associated with PS perceptions in some groups of respondents), which contradicts the available studies showing the role of parents' relationships in the formation of perceptions [17]. It does, however, point to the role of inclusion in the family system, the presence of systemic social support as a protective factor in the perception of the parental role stressor. Baseline beliefs have not been identified as determinants of general perceptions of parental role stressors, but some of them may influence the parental stress perceptions of a group of young people oriented toward having many children.

Determinants analysis of parenting stressor perceptions taking into account reproductive plans allows observing the presence of a system of producing (determining) and secondary (acting as conditions) factors of parental role stressor. Among both the former and the latter, however, there are statistically significantly

strong and numerous associations of PS parameters with subjective factors in all three groups. Common for all groups was the negative relationship of parental stress parameters with satisfaction, which fully correlates with the studies of foreign scientists indicating negative aspects of parenting [1, 2].

The group of hypothetical parents with few children, among whom the majority grew up in complete families and they have the highest percentage of no siblings among all groups, represent the parental role as moderately stressful, having a resource in the form of receiving positive emotions, pleasure from parenting. The factor of age acts as objective determinants of PS perceptions, which suggests that critical but anxious attitudes to parenting and, accordingly, attitudes to having few children during maturity are fixed. Female gender and education may be contributing factors to PS perceptions in this group. Among the subjective determinants of parenting stressor is a combination of different attitudes to the intensity of parenting, as well as low evaluation of family relationships. The combination of attitudes to the mother's primacy («essentialism») and attitudes to the deficit of nurturing pleasure («satisfaction») or to the need to stimulate development («stimulation») most often plays a significant role in the system of producing subjective factors that reinforce PS. This forms a certain psychological portrait of the family that exists in this group and is associated with PS, with a hyper-responsible mum who feels the need to develop the child and does not enjoy doing so.

The group of respondents with orientation to large families, who revealed the lowest expression of parental role stressor, were predominantly brought up in a full family, and a significant part of them – in large families. Their objective determinants of their PS perceptions are the experience of incomplete education. This is probably considered in this group as a loss of a resource against PS. As in the previous group, belonging to the female sex has an enhancing effect here, while only the male sex is associated with a decrease in PS. The obtained results are consistent with the data of other scientists [1, 7], which is relevant in the case of the emerging trend to change the situation and to greater involvement of men in the process of child upbringing and, consequently, their greater exposure to parental stress. The subjective determinants are exclusively attitudes towards upbringing, among which the parameter of essentialism and the feeling of mothers' primary responsibility for upbringing a child are the leading ones. This may be a result of fear of social support loss, as reported, for example, by women with children [14].

In this group, the correlation of PS parameters with the assessment of relations in the family system (child-parental and family relations in general) is also observed, which is a condition for strengthening PS in the perceptions of young people. The literature partly highlights family conflict [8] and characteristics of parental families as stressors [19], and in our case this may emphasise the role of subjective evaluation of parental family upbringing experiences. The influence of basic beliefs over PS indicators was revealed only in this group of respondents, which, in our opinion, may indicate the role of personal factors in the formation of perceptions of parenting and requires further study.

Almost complete absence of objective determinants of parental role stressor was revealed only in the group of people with childlessness orientation. This group has more male respondents who are not in a relationship and the highest expression rates of perceived PS, which may be influenced (as an objective condition) by the position of an only child in the family. The inability to derive pleasure and fulfilment from the upbringing process is a derived factor for PS symptoms, and the expression of other upbringing attitudes may influence perceptions of the stressor of the parental role. Of interest is the established negative effect of unformed child-centrism attitudes and the need to stimulate children on the increase in signs of parental stress. This fact suggests an internal conflict among these respondents: on the one hand - unwillingness to make upbringing of a child a life priority and spend personal resources on it, on the other hand - understanding the necessity of it. This situation, in our opinion, can lead to feelings of guilt, and the chosen orientation towards childlessness can be considered as a way out of the conflict.

The limitations of the study are some overview of the highlighted subjective factors of disclosure of determinants of perceptions of parental stress, which is due to the exploratory nature of the study. The age of respondents imposes restrictions upon generalising the findings to the entire population of young people without children (e.g. over 30), but it allows us to identify statistically significant patterns relevant to Generation Z youth. We see a full account of the determinants of perceptions of parental stress as a prospect for further studies of this topic.

## CONCLUSIONS

As a result of theoretical analyses, the negative impact of parental stress and the tendency towards intense parental role fulfilment upon the mental health of parents and children in terms of creating obstacles to normative maturation and mental development has been observed.

The empirical study allowed us to identify a set of factors that can influence the increase of parental role stressor in the perceptions of potential parents and, accordingly, increase the risk of parental behaviour unfavourable for their own mental and physical health and children's health.

Young people as potential parents perceive the parental role in terms of its «intensity» and labour-intensiveness. Generally, young people's perceptions of the potential stress of parenting are determined by a complex of contextual «objective» (age, status of personal relationships, education) and personal «subjective» (assessment of family relationships, attitudes

to the need to stimulate child development, essentialism and getting satisfaction from upbringing) factors, but subjective factors make the greatest contribution.

The level of stressor of parental role statistically significantly differs in the perceptions of young people with different orientation to childbearing, which can be considered as one of the conditions for the formation of fertile attitudes. The level of parenting stressor is determined by a system of determinant factors. As general preconditions of PS (independent of plans for childbearing) are attitudes to the intensity of upbringing, which, apparently, are formed as a generalisation of children's family and social experience. The attitude of a deficit of pleasure in upbringing plays a fundamental role here.

In the group of persons with a child-free orientation, there is almost complete absence of determination of stressor parenting by objective characteristics, and the high level of PS in young people's ideas is conditioned by the attitude to the impossibility of self-fulfilment and enjoyment from upbringing children. The choice of childlessness orientation may be a way of resolving the conflict between unformed attitudes to the priority of a child and the pressure of social requirements for upbringing.

Similarity of determinants of PS, moderate level of which is predicted in the future, in groups of respondents with childbearing orientation was revealed. The main parameters are essentialism, reflecting the unequal distribution of responsibilities for upbringing and development of children between mother and father, excessive level of mother's responsibility for this. In the group of young people with orientation having few children, the increase in PS and its symptoms is determined by a set of attitudes including essentialism, as well as the evaluation of family relationships.

Thus, the predominant determination of perceptions of parental role stressor by subjective factors as opposed to objective factors was observed. The specificity of the determination of ideas about parental stress depending on the orientation towards childbearing has been revealed.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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## PHARMACOLOGY AND PHARMACY

# DEVELOPMENT AND VALIDATION OF A METHOD FOR THE QUANTITATIVE DETERMINATION OF MONOAMINE NEUROTRANSMITTERS AND THEIR METABOLITES IN RAT BRAIN TISSUE USING HPLC-MS/MS

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## ABSTRACT

***Background.*** *Determining changes in the content of monoamine neurotransmitters and their metabolites in brain structures is a necessary part of studying the pharmacodynamics of antiparkinsonian drugs. A method for the joint determination of norepinephrine, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, vanillylmandelic acid in rat brain tissue has not previously been developed.*

***The aim of the study.*** *Development and validation of a method for quantitative determination of noradrenaline, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and vanillylmindalic acid in rat brain tissue by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).*

***Materials and methods.*** *A method for determining monoamine mediators and their metabolites was developed using the HPLC-MS/MS method. Brain tissue homogenates were prepared using a mechanical hand-operated homogenizer. The effect of various antioxidants on the stability of norepinephrine, adrenaline, dopamine and 3,4-dihydroxyphenylacetic acid in the test samples was studied.*

***Results.*** *Chromatographic separation of sample components was carried out using two Synergi Max RP (20 × 2.0 mm, 2.5 µm) and Synergi Fusion RP 80Å (250 × 4.6 mm, 4 µm) chromatographic columns. Elution was carried out in a gradient mode using a mobile phase based on methanol and a 0.1 % solution of formic acid in water. To prepare homogenate batches, the samples were diluted with a solution of internal standards in methanol. A 5 % aqueous solution of ascorbic acid was chosen as an antioxidant stabilizer.*

***Conclusion.*** *The developed methodology has been fully validated and meets the requirements of Russian and international guidelines. The chosen stabilization method allows samples of brain homogenates to be stored for 30 days after collection.*

***Key words:*** *HPLC-MS/MS, monoamine neurotransmitters, brain tissue, sample stabilization*

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# РАЗРАБОТКА И ВАЛИДАЦИЯ МЕТОДИКИ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ МОНОАМИНОВЫХ НЕЙРОМЕДИАТОРОВ И ИХ МЕТАБОЛИТОВ В ТКАНЯХ МОЗГА КРЫС С ПОМОЩЬЮ ВЭЖХ-МС/МС

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## РЕЗЮМЕ

***Обоснование.*** *Определение изменения содержания моноаминовых нейромедиаторов и их метаболитов в структурах головного мозга является необходимой частью изучения фармакодинамики противопаркинсонически лекарственных средств. Методика совместного определения норадреналина, адреналина, допамина, серотонина, 5-гидроксииндол-3-уксусной кислоты, 3,4-дигидроксифенилуксусной кислоты, гомованилиновой кислоты, ванилилминдальной кислоты в тканях мозга крыс ранее не была разработана.*

***Цель исследования.*** *Разработка и валидация методики количественного определения норадреналина, адреналина, допамина, серотонина, 5-гидроксииндол-3-уксусной кислоты, 3,4-дигидроксифенилуксусной кислоты, гомованилиновой кислоты, ванилилминдальной кислоты в тканях мозга крыс с помощью высокоэффективной жидкостной хроматографии в сочетании с тандемной масс-спектрометрией (ВЭЖХ-МС/МС).*

***Методы.*** *Методика определения моноаминовых медиаторов и их метаболитов разработана с применением метода ВЭЖХ-МС/МС. Гомогенаты тканей мозга готовились с помощью механического ручного гомогенизатора. Изучено влияние различных антиоксидантов на стабильность норадреналина, адреналина, допамина и 3,4-дигидроксифенилуксусной кислоты в испытуемых образцах.*

***Результаты.*** *Хроматографическое разделение компонентов пробы осуществлялось с помощью двух хроматографических колонок Synergi Max RP (20 × 2,0 мм, 2,5 мкм) и Synergi Fusion RP 80Å (250 × 4,6 мм, 4 мкм). Элюирование проводили в градиентном режиме с применением подвижной фазы на основе метанола и 0,1%-го раствора муравьиной кислоты в воде. Для подготовки проб гомогенатов использовалось разведение образцов раствором внутренних стандартов в метаноле. В качестве стабилизатора-антиоксиданта был выбран 5%-й водный раствор аскорбиновой кислоты.*

***Заключение.*** *Разработанная методика прошла полную валидацию и соответствует требованиям российских и международных руководств. Выбранный способ стабилизации позволяет хранить образцы гомогенатов мозга в течение 30 дней после отбора.*

***Ключевые слова:*** *ВЭЖХ-МС/МС, моноаминовые нейромедиаторы, ткани мозга, стабилизация образцов*

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## INTRODUCTION

Parkinson's disease is a neurodegenerative disease in which there is a decrease in the number of dopaminergic neurons in the substantia nigra, which causes a decrease in the concentration of dopamine (Dop) in the striatum. This causes the classic motor symptoms: rigidity, posture disturbances, akinesia, tremor, and bradykinesia. Most of the existing models of this disease involve the use of rats as experimental animals [1]. When studying the pharmacodynamics of new antiparkinsonian medicinal products (MPs), quantification of dopamine and its major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC, 3,4-dihydroxyphenylacetic acid) and homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid; HVA, homovanillic acid) in the striatum is required. One of the promising groups of MPs used in the treatment of Parkinson's disease are inhibitors of the enzyme MAO-B, which selectively catalyses the oxidation of dopamine. In order to study the effect of these MPs on MAO-A activity, it is necessary to measure the concentration of noradrenaline (NA, noradrenaline), serotonin (5HT, 5-hydroxytryptamine) and its metabolite (5-hydroxyindole-3-yl)-acetic acid (5HIAA, 5-hydroxyindoleacetic acid) [1, 2]. To control the correctness of brain and striatum sampling and the absence of contamination by blood and other tissue particles, it is necessary to monitor the content of adrenaline (Adr) and vanillylmindelic acid (2-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-acetic acid; VMA, vanillylmandelic acid), which should not normally be detected in these samples [3-7].

High-performance liquid chromatography with spectrophotometric (HPLC-UV) [8], electrochemical (HPLC-EM) [9-12] and tandem mass spectrometric detector (HPLC-MS) [2, 5-7, 13-20] was used for quantitative determination of the above analytes in biological objects. However, the methodology for co-analysing all eight substances studied together in brain structures has not been previously published. The process of developing this technique is complicated by the fact that noradrenaline, adrenaline, dopamine and DOPAC contain a pyrocatechin fragment in their structure, which contributes to their rapid oxidation in samples as a result of interaction with endogenous substances and air oxygen [4, 21]. The addition of antioxidant solutions to the samples is required to prevent degradation of these compounds. The necessity of using a stabiliser is indicated only in the studies of J. Lu et al. [2], J. Thomas et al. [8], G. Cannazza et al. [12], C. Ji et al. [15], A. Kovac et al. [17]. Consequently, the choice of optimal conditions for stabilisation and storage of selected rat brain tissues is also relevant to ensure the reliability of the results of preclinical studies.

## THE AIM OF THE STUDY

Development and validation of a method for the combined quantitative determination of noradrenaline, adrenaline, dopamine, serotonin, 5-hydroxyindole-3-acetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and vanillylmindalic acid in rat brain tissue.

## MATERIALS AND METHODS

### Study design

The first stage of the study involved the selection of optimal conditions for homogenate sample preparation as well as parameters for chromatographic-mass spectrometric determination. The selection of an antioxidant stabiliser was then performed to prevent degradation of noradrenaline, adrenaline, dopamine and DOPAC. The matrix effects of the methodology were further evaluated. Based on the results obtained, correction of the volumetric ratio of solvent to tissue in the preparation of homogenates was performed. In the next step, a full validation of the bioanalytical methodology was performed. It was then tested by analysing striatum samples from six intact Wistar male line rats weighing 362 ± 25 g (mean ± standard deviation (SD, standard deviation)). The study was approved by the Ethical Committee of the Yaroslavl State Medical University of the Ministry of Health of Russian Federation (Minutes No. 2 dated March 23, 2023).

### Equipment

Method development and validation were performed on an HPLC-MS/MS system comprising a QTRAP5500 hybrid tandem mass spectrometer (SCIEX, Canada) and a 1260 Infinity chromatograph (Agilent, USA) (G1312B pump, G1329B autosampler with G1330B thermostat, G1316A column thermostat).

### Reagents

Methanol (Cat. No. 1060352500; Merck KGaA, Germany) and formic acid (Cat. No. A117-50; Thermo Fisher Scientific, USA) of «HPLC-MS-Grade» quality were used to prepare the mobile phase. Substances of ascorbic acid (c.p. (chemically pure); cat. No. 160003; JSC «Lenreaktiv», Russia), sodium sulfite (r.g. (reagent grade); cat. No. 130231; JSC «Lenreaktiv», Russia), sodium thiosulfate pentahydrate (cat. No. SO07270500; Scharlau, Spain), sodium pyrosulfite (p. (pure); cat. No. 8.06.00804; JSC «Khimreaktivsnab», USA) were tested as antioxidants. Secondary standard samples produced by Sigma Aldrich (USA) were used as standard samples of the substances to be determined: noradrenaline (Cat. No. A7257-1G), adrenaline hydrochloride (Cat. No. E4642-5G), serotonin (Cat. No. 14927-25MG), dopamine hydrochloride (Cat. No. H8502-5G), (5-hydroxyindole-3-yl)-acetic acid (Cat. No. H8876-1G), 3,4-dihydroxyphenylacetic acid (Cat. No. 850217-1G), homovanilic acid (Cat. No. H1252-1G), vanillylmindalic acid (Cat. No. H0131-1G). The 3,4-dihydroxybenzylamine hydrobromide (3,4-DHBA) substance (Cat. No. 858781-1G) and the pharmacopoeial standard sample of sotalol (USP; Cat. No. 1617408) were used to prepare the internal standards (IS) solution (Fig. 1).

**Chromatography-mass spectrometric determination technique**

The chromatographic separation was performed under gradient mode using two columns Synergi Max RP (20 × 2.0 mm, 2.5 μm) and Synergi Fusion RP 80Å (250 × 4.6 mm, 4 μm) using 0.1 % aqueous formic acid solution and methanol as mobile phase components (Table 1). These reversed-phase columns had additional

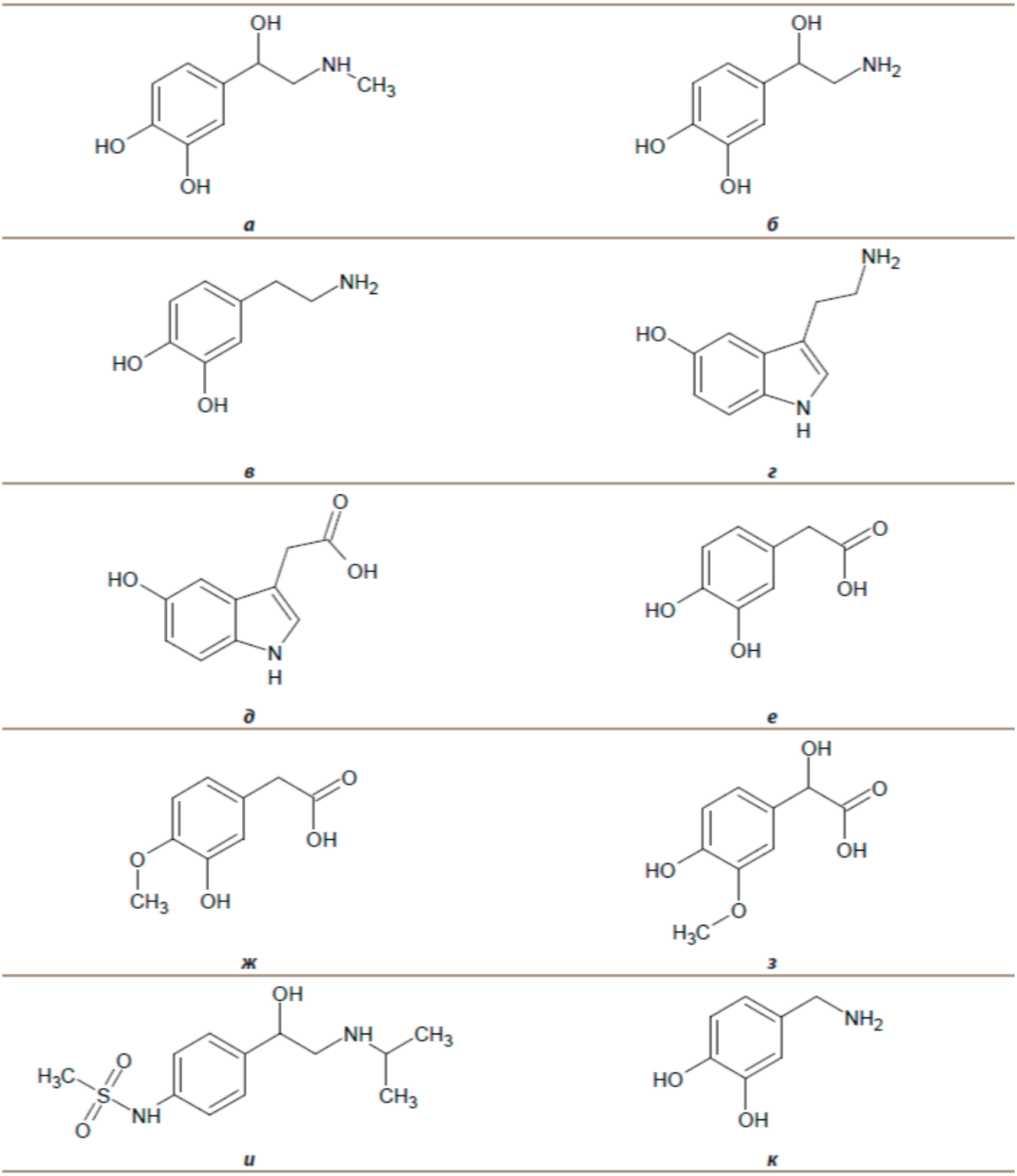


***FIG. 1.***
*Structural formulae of adrenaline (а), noradrenaline (б), dopamine (в), serotonin (г), (5-hydroxyindole-3-yl)-acetic acid (д), 3,4-dihydroxyphenylacetic acid (е), homovanillic acid (ж), vanillylmindalic acid (з) and internal standards of sotalol (и) and 3,4-dihydroxybenzylamine (к)*

hydrophilic functional groups required for retention of polar catecholamines. The thermostat temperature of the columns was 40 °C.

Detection was performed in MRM (multiple reaction monitoring) mode (Table 2) using electrospray ionisation (ESI, electrospray ionization). NA, Adr, Dop, 5HT, 5HIAA, and 3,4-DHBA were determined in positive polarity; DOPAC, HVA, and VMA were determined in negative polarity. Sotalol was detected in both polarities: positive for 5HT and 5HIAA concentration calculation; negative for DOPAC, HVA and VMA concentration calculation. This compound was used because of its structural similarity to catecholamines and the closeness of its retention time (10.4 min) to that of 5HT (10.1 min), 5HIAA (12.4 min), DOPAC (11.9 min), HVA (12.9 min) and VMA (10.8 min). 3,4-DHBA was used as an internal standard for the determination of norepinephrine, adrenaline and dopamine. Its choice is based on previously published methods for quantification of these analytes

**TABLE 1**

**GRADIENT ELUTION PARAMETERS**

| Time, min | Flow rate, µl/min | A, % | B, % |
|---|---|---|---|
| 0.0 | 650 | 98 | 2 |
| 2.0 | 650 | 98 | 2 |
| 9.0 | 650 | 20 | 80 |
| 14.1 | 650 | 20 | 80 |
| 14.5 | 1000 | 20 | 80 |
| 15.0 | 1000 | 20 | 80 |
| 15.1 | 1000 | 98 | 2 |
| 19.0 | 1000 | 98 | 2 |
| 19.2 | 650 | 98 | 2 |
| 21.0 | 650 | 98 | 2 |

**Note.** Mobile phase: A – 0.1 % aqueous solution of formic acid; B – methanol.

### Method validation parameters

Full validation of the method was performed in accordance with the requirements of the guidelines for validation of bioanalytical methods (M10) of the International Council on Harmonisation (ICH) [22], FDA (Food and Drug Administration) guidelines [23], guidelines (Volume 1) of the Scientific Centre for Expert Evaluation of Medicinal Products of the Ministry of Health of Russia (SCEEMP) [24] and Decision of the Council of the Eurasian Economic Commission (EAC) No. 85 (Annex 5) [25] related to chromatographic methods. Model homogenate mixtures were prepared from whole brain and striatum samples of Wistar rats. These objects were placed in a pre-calibrated tube of a manual homogeniser

**TABLE 2**

**MASS SPECTROMETRIC DETECTION PARAMETERS**

| No. | Analyte | Polarity | ESI voltage, V | MRM junction | | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Q1 | Q3 | | | | |
| 1 | Adr | + | 4500 | 184.0 | 77.0 | 60 | 10 | 45 | 25 |
| 2 | NA | + | 4500 | 170.0 | 77.0 | 60 | 10 | 40 | 25 |
| 3 | Dop | + | 4500 | 154.0 | 119.0 | 60 | 10 | 35 | 25 |
| 4 | 5HT | + | 4500 | 177.0 | 160.0 | 60 | 10 | 30 | 25 |
| 5 | 5HTAA | + | 4500 | 192.0 | 146.0 | 60 | 10 | 20 | 13 |
| 6 | DOPAC | – | –4500 | 167.0 | 123.0 | –60 | –10 | –10 | –30 |
| 7 | HVA | – | –4500 | 181.0 | 122.0 | –60 | –10 | –20 | –25 |
| 8 | VMA | – | –4500 | 197.0 | 137.0 | –60 | –10 | –25 | –30 |
| 9 | 3.4-DHBA | + | 4500 | 140.0 | 77.0 | 60 | 10 | 25 | 25 |
| 10 | Sot | + | 4500 | 273.0 | 133.0 | 60 | 10 | 80 | 13 |
| | | – | –4500 | 271.0 | 174.0 | –60 | –10 | –40 | –16 |

**Note.** Sot – sotalol; DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – collision cell exit potential.

**TABLE 3**

**CALIBRATION AND QUALITY CONTROL SAMPLE CONCENTRATIONS**

| Designation | Concentration, ng/g | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NA | Adr | Dop | 5HT | DOPAC | 5HTAA | HVA | VMA |
| K1 (LLOQ) | 50 | 50 | 1250 | 75 | 200.0 | 150 | 80 | 67.50 |
| K2 | 100 | 100 | 2500 | 150 | 400.0 | 300 | 160 | 135.00 |
| K3 | 200 | 200 | 5000 | 300 | 800.0 | 600 | 320 | 270.00 |
| K4 | 300 | 300 | 7500 | 450 | 1200.0 | 900 | 480 | 405.00 |
| K5 | 400 | 400 | 10000 | 600 | 1600.0 | 1200 | 640 | 540.00 |
| K6 | 600 | 600 | 15000 | 900 | 2400.0 | 1800 | 960 | 810.00 |
| K7 | 900 | 900 | 22500 | 1350 | 3600.0 | 2700 | 1440 | 1215.00 |
| K8 | 1200 | 1200 | 30000 | 1800.0 | 4800 | 3600 | 1920 | 1620.00 |
| LQC | 150 | 150 | 3750 | 225.0 | 600 | 450 | 240 | 202.50 |
| MQC | 500 | 500 | 12500 | 750.0 | 2000 | 1500 | 800 | 675.00 |
| HQC | 975 | 975 | 24375 | 1462.5 | 3900 | 2925 | 1560 | 1316.25 |
| Dil | 1800 | 1800 | 45000 | 2700.0 | 7200 | 5400 | 2880 | 2430 |

**Note.** Dil – concentration to estimate dilution effect; K – calibration concentration.

and homogenised after adding solvent in the required volume. After centrifugation, a standard solution of the analyte mixture was added to the supernatant fluid at a rate of 10 µl of standard solution per 190 µl of supernatant. To study the selectivity of the technique (series 1 in Table 6), as well as the effect of sample dilution, brain samples stored for 2 years at a temperature no higher than -20 °C were used, in which there was no analytical signal of the analytes. Linearity was assessed at 8 concentration levels (K1-K8), accuracy and coefficient of variation at 4 concentration levels (at the lower limit of quantitation (LLOQ, lower limit of quantitation), lower (LQC, lower quality control), middle (MQC, middle quality control) and higher (HQC, higher quality control) quality control levels), and dilution effect at the same concentration level (Dil) (Table 3).

## RESULTS

At the initial stage of development, methanol was chosen as a solvent for the preparation of homogenates, since when water was used, the NA peak was absent on the chromatograms (retention time $t_R$ = 4.7 min), and when acetonitrile was used, its signal-to-noise ratio (S/N, signal/noise) was 10 times lower (106/1). Samples were prepared using a manual homogeniser by adding solvent at a rate of 3 µl of solvent per 1 mg of brain tissue[1]. The homogenates were then centrifuged for 5 min at 10000 rpm and 10 µl of methanol standard solution with K4 concentration was added to 190 µl of supernatant (Table 3). A 120 µl methanol solution of a mixture of internal standards of sotalol and 3,4-DHBA was added to 50 µl of the obtained sample. The mixture was stirred on a shaker for 30 s and then centrifuged for 5 min at 10000 rpm.

In the next step, antioxidant (AO) solution selection was performed by studying the short-term stability (STS, short-term stability) of NA, Adr, Dop and DOPAC in brain homogenate samples, as well as the stability of these analytes in prepared samples in autosampler (ASS, autosampler stability). Aqueous solutions of ascorbic acid, sodium sulfite, sodium metabisulfite, and sodium thiosulfate at concentrations of 5 % and 10 % were used as stabiliser [21]. Antioxidant solution was added at a rate of 10 µl of solution per 50 µl of homogenate. The test results are summarised in Table 4.

[1] The tissue sample was weighed in a pre- calibrated homogeniser tube and then methanol was added to it in the desired amount: for example, if the tissue mass was 100 mg, 300 µl of methanol was added to it.

**TABLE 4**

**ANTIOXIDANT STABILISER SELECTION RESULTS**

| | | | Antioxidant | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Without AO ($n$ = 2) | Ascorbic acid | | $Na_2S_2O_3$ | | $Na_2SO_3$ | |
| | | | | 5 % ($n$ = 2) | 10 % ($n$ = 2) | 5 % ($n$ = 2) | 10 % ($n$ = 2) | 5 % ($n$ = 2) | 10 % ($n$ = 2) |
| Homogenate in methanol 1:3 (mass/vol.) | NA | ASS (+4 °C, 24 hours) | **56.24** | 109.55 | 100.12 | 87.24 | 96.82 | **74.82** | **71.43** |
| | | STS (room temperature, 24 h) | **49.02** | 91.02 | 114.71 | 100.48 | 103.19 | **78.18** | **77.61** |
| | Adr | ASS (+4 °C, 24 hours) | **62.01** | 97.66 | 101.95 | **74.99** | **79.85** | **71.44** | **48.92** |
| | | STS (room temperature, 24 h) | **59.52** | 102.19 | 112.68 | **34.19** | **62.90** | **22.20** | **38.45** |
| | Dop | ASS (+4 °C, 24 hours) | **39.28** | 105.03 | 96.62 | 92.53 | N/A | **83.01** | N/A |
| | | STS (room temperature, 24 h) | **35.21** | 101.39 | 95.96 | **54.65** | N/A | **48.12** | N/A |
| | DOPAC | ASS (+4 °C, 24 hours) | **78.26** | 107.21 | 97.88 | 93.37 | 87.44 | 94.24 | 90.94 |
| | | STS (room temperature, 24 h) | **72.42** | 107.52 | 100.55 | **78.27** | 87.18 | 92.37 | 94.51 |
| Homogenate in methanol 1:7 (mass/vol.) | NA | ASS (+4 °C, 24 hours) | 90.00 | 90.15 | – | – | – | – | – |
| | | STS (room temperature, 24 h) | **75.45** | 97.35 | – | – | – | – | – |
| | Adr | ASS (+4 °C, 24 hours) | **70.73** | 95.98 | – | – | – | – | – |
| | | STS (room temperature, 24 h) | **23.17** | 100.57 | – | – | – | – | – |
| | Dop | ASS (+4 °C, 24 hours) | 88.31 | 103.93 | – | – | – | – | – |
| | | STS (room temperature, 24 h) | **49.93** | 98.61 | – | – | – | – | – |
| | DOPAC | ASS (+4 °C, 24 hours) | 95.55 | 97.28 | – | – | – | – | – |
| | | STS (room temperature, 24 h) | 90.24 | 95.10 | – | – | – | – | – |

**Note.** N/A – no chromatographic peak of the analyte.

The addition of sodium sulfite and sodium thiosulfate solutions failed to prevent oxidation of all analytes (Table 4). For instance, when $Na_2SO_3$ solutions were used, only the DOPAC concentration fell within the required range of 85–115 % of the initial value. The use of $Na_2S_2O_3$ solution at a concentration of 5 % prevented the oxidation of noradrenaline as well as dopamine and DOPAC in the prepared samples in the autosampler. The chromatographic peak of Dop was not detected in samples with the addition of 10 % solutions of $Na_2S_2O_3$ and $Na_2SO_3$. When sodium metabisulfite solutions were added to the methanol homogenates, this salt precipitated, so these samples were not analysed. Only when ascorbic acid was used the concentrations of all analytes in the short-term stability and autosampler stability tests were in compliance. For further testing, an aqueous solution of ascorbic acid at the lowest concentration of 5 % was chosen to minimise the risk of contaminating the chromatography column, ion source and ion optics of the mass spectrometer with excessive amounts of this substance.

At the next stage of the study, matrix effects were studied. Freshly sampled rat brain homogenates, freshly sampled striatum homogenates, and brain homogenates stored for 2 years at a temperature not exceeding -20 °C, obtained from 6 different animals, were used

**TABLE 5**

**EVALUATION OF THE MATRIX EFFECT IN THE DETERMINATION OF ANALYTES IN BRAIN HOMOGENATES**

| | Analytes | | Homogenate in a ratio of 1:3 | Homogenate in a ratio of 1:7 |
|---|---|---|---|---|
| CV (NMF), % | NA | LQC | 13.48 | 11.39 |
| | | HQC | 14.09 | 4.80 |
| | Adr | LQC | 13.94 | 7.46 |
| | | HQC | 12.76 | 10.56 |
| | Dop | LQC | 8.95 | 6.07 |
| | | HQC | 5.15 | 7.29 |
| | 5HT | LQC | 13.98 | 5.93 |
| | | HQC | **17.69** | 4.37 |
| | 5HIAA | LQC | 11.14 | 9.77 |
| | | HQC | 6.25 | 5.23 |
| | DOPAC | LQC | **26.82** | 7.89 |
| | | HQC | **21.38** | 7.49 |
| | HVA | LQC | 11.10 | 5.37 |
| | | HQC | 11.95 | 8.65 |
| | VMA | LQC | 10.90 | 8.37 |
| | | HQC | 10.31 | 5.07 |
| Peak area ratio «analyte/ internal standard» (mean value) | 5HT (brain homogenate) (*n* = 4) | LQC | 0.183 | 0.135 |
| | | HQC | 1.464 | 0.956 |
| | 5HT (striatum homogenate) (*n* = 2) | LQC | 0.142 | 0.132 |
| | | HQC | 1.021 | 0.940 |
| | DOPAC (brain homogenate) (*n* = 4) | LQC | 4.635 | 2.223 |
| | | HQC | 33.255 | 15.538 |
| | DOPAC (striatum homogenate) (*n* = 2) | LQC | 2.701 | 2.115 |
| | | HQC | 22.502 | 15.327 |

**Note.** CV (NMF) – coefficient of variation for normal matrix factorization.

for the preparation of model mixtures. According to the requirements of Russian and foreign guidelines for validation of bioanalytical techniques [23-25], each of 6 samples were analysed at the level of LQC and HQC concentrations (Table 3), as well as a sample of each homogenate without the addition of a standard to subtract the signal of endogenous substances (Table 5).

At the initial conditions of sample preparation, the value of the coefficient of variation (CV) of the normalised matrix factorization (NMF) in the determination of 5HT and DOPAC exceeded the permissible limit of 15 % (Table 5). To reduce matrix effects, the ratio of methanol to brain tissue in the preparation of homogenates was adjusted: the sample was prepared at a rate of 7 µl of solvent per 1 mg of tissue. Under these sample preparation conditions, the CV (NMF) result was in compliance for all analytes. Afterwards, the stability of NA, Adr, Dop and DOPAC was re-tested in samples of methanol homogenate prepared in a 1:7 ratio using the previously selected 5 % aqueous ascorbic acid solution (Table 4). The concentrations of all analytes in the ASS and STS tests were within the acceptable range of 85.0–115.0 % of the initial value.

Therefore, in order to quantify the concentration of the studied substances in brain tissue, the homogenate was prepared manually at a ratio of 1:7 (tissue weight/ volume of methanol). The sample was then centrifuged for 5 min at 10000 rpm. And stabilised with an aqueous solution of ascorbic acid at a concentration of 5 % at a rate of 10 µl of solution per 50 µl of supernatant. Thereafter, 120 µl of methanol solution of internal standards was added to 60 µl of the mixture, stirred for 30 s and centrifuged for 5 min at 10000 rpm. The supernatant fluid was transferred to a microtiter plate and analysed by HPLC-MS/MS.

After selecting the final sample preparation conditions and chromatography-mass spectrometric determination, a complete validation of the methodology was performed. The analytical range for Adr and NA was 50–1200 ng/g, for Dop – 1.25–30.00 µg/g, for 5HT – 75–1800 ng/g,

for DOPAC – 200–4800 ng/g, for 5HIAA – 150–3600 ng/g, for HVA – 80–1920 ng/g, and for VMA – 67.5–1620 ng/g. The dependence of the analyte/internal standard peak area ratio on the concentration of each compound was linear. In evaluating the selectivity of the method using brain homogenate samples stored at a temperature not exceeding -20 °C for 2 years, the area of chromatographic peaks in blank matrices for analytes did not exceed 20 % of the peak area in LLOQ samples, for internal standards of sotalol, for internal standards of 3,4-DHBA, the area of chromatographic peaks did not exceed 5 % of the peak area in LLOQ samples (Fig. 2, 3).

The mean values of the calculated determination concentrations of all studied compounds were within 85–115 % of the nominal value for LQC, MQC and HQC concentration levels, within 80–120 % for LLOQ concentration level,

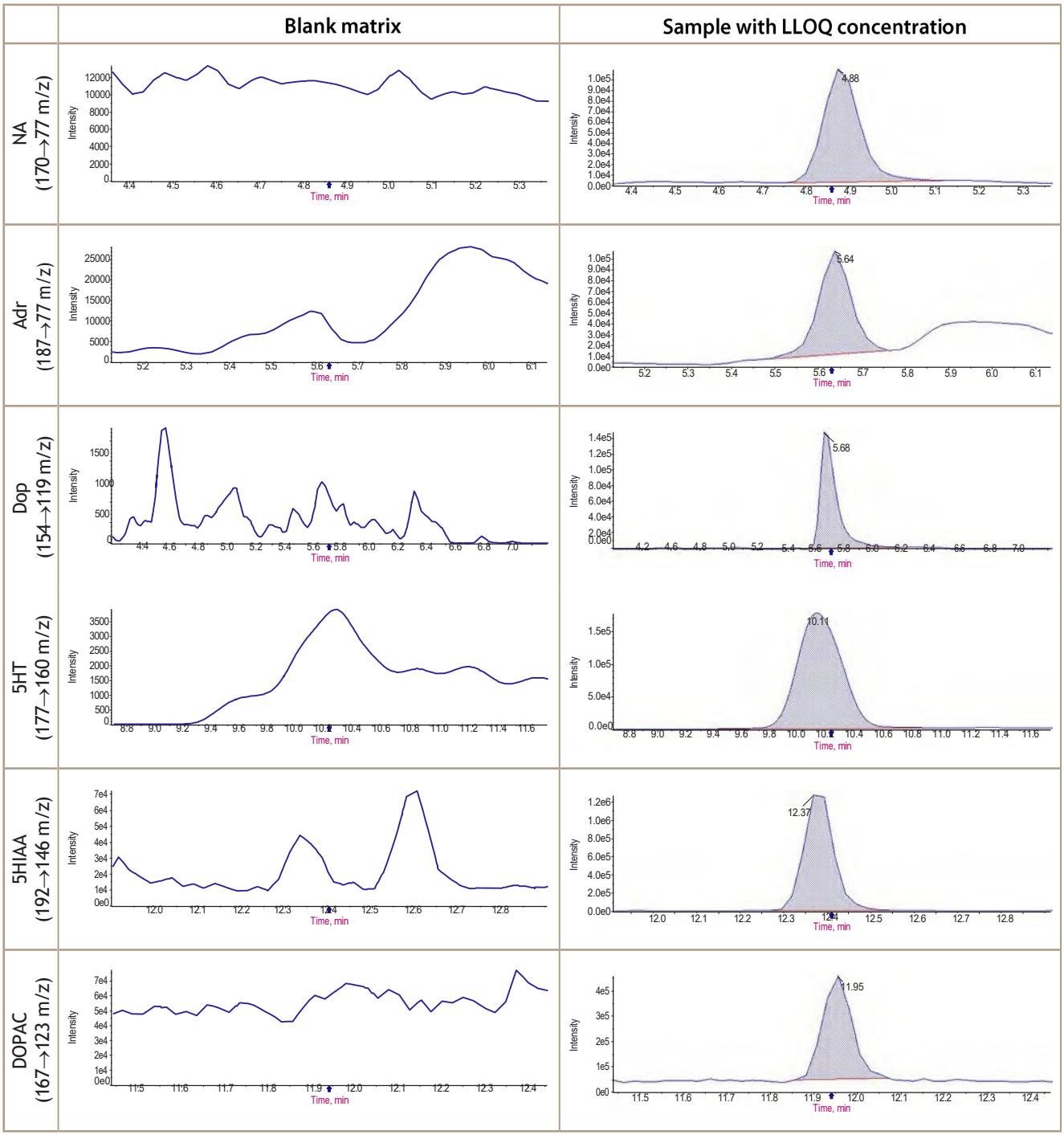


***FIG. 2.***
*Examples of MRM chromatograms of blank matrix and sample with added standard at LLOQ level (analytes - NA, Adr, Dop, 5HT, 5HIAA, DOPAC)*

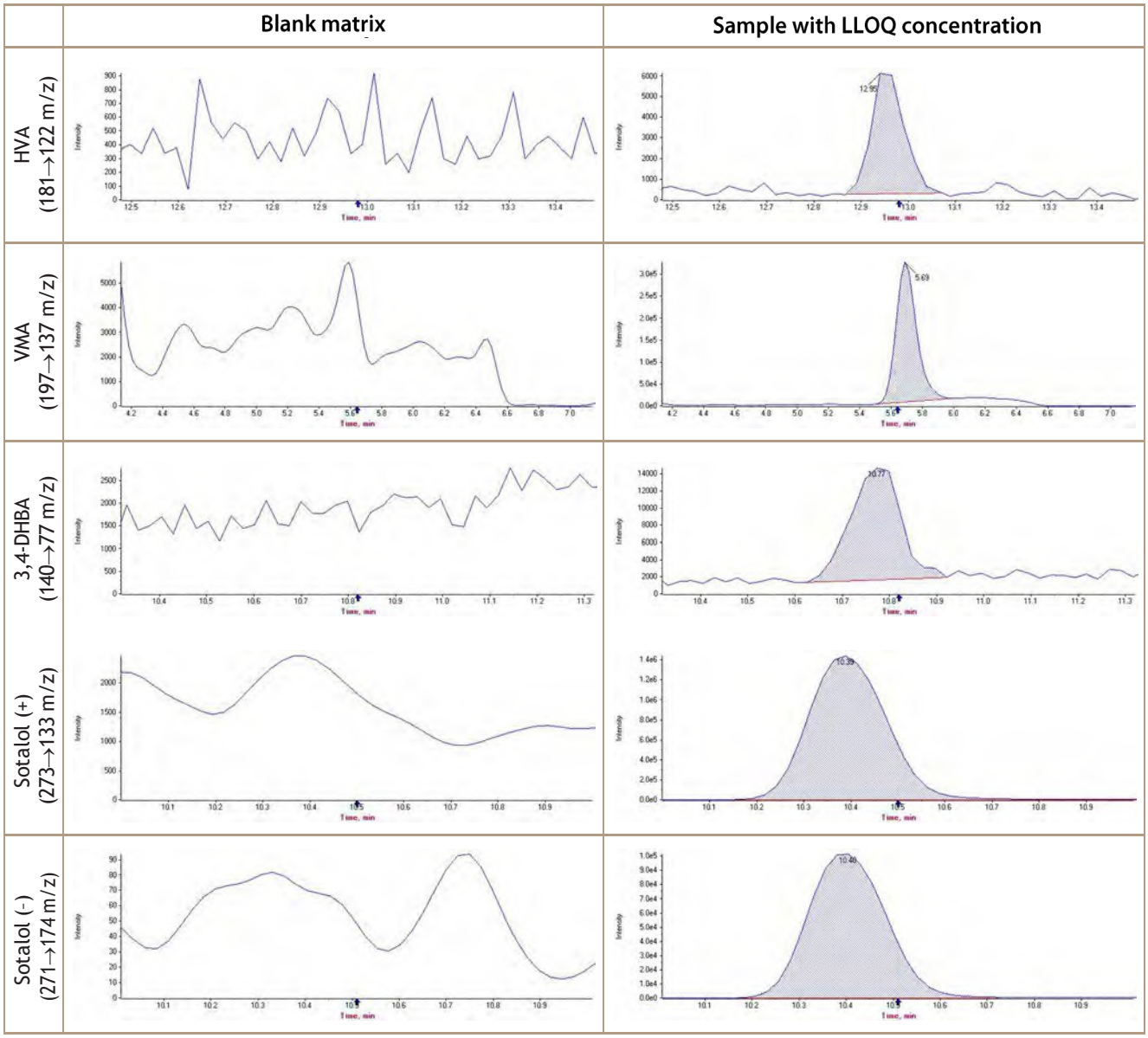


***FIG. 3.***
*Examples of MRM chromatograms of blank matrix and sample with added standard at LLOQ level (analytes - HVA, VMA, 3,4-DHBA (BC), sotalol (BC))*

including the assessment of the selectivity of the methodology (Table 6, series 1). The coefficient of variation of the calculated concentrations did not exceed 15 %. In this case, the result of analytical series 2 and 3 (Table 6), performed using samples of freshly collected brain homogenates, was calculated considering the endogenous content of analytes in samples without the addition of the standard. Double dilution of samples with the content of the studied substances exceeding the analytical range (Dil; Table 3) with blank matrix did not affect the metrological characteristics of the method: the value of calculated concentrations of analytes was within the range of 89.76–94.99 % of the nominal value, the CV value – within the range of 2.77–5.88 % (Table 6). There was no carryover of analytes and internal standards from the previous sample.

The selected antioxidant solution provides stability of analytes in homogenate samples during 24 h storage at room temperature, 3 freezing/thawing cycles (FTS, freezing/thawing stability), 30 days storage in a freezer at a temperature not exceeding -20 °C (LTS, long-term stability), as well as stability in prepared samples in an autosampler during 48 h at +4 °C (Table 7).

The developed method was tested by analyzing striatum samples obtained from 6 intact male Wistar line rats. Brain samples were chilled with liquid nitrogen immediately after collection, and the striatum was extracted. Homogenization and addition of stabilizer solution was performed no later than 20 min after sampling. The results of NA, Adr, Dop, 5HT, DOPAC, 5HIAA, VMA, and HVA determinations are presented in Table 8.

**TABLE 6**

**VALIDATION RESULTS OF THE DEVELOPED METHODOLOGY**

| Indicators | | Adr | | NA | | Dop | | 5HT | | 5HIAA | | DOPAC | | HVA | | VMA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Selectivity | | Interference in the retention times of analytes in blank matrices did not exceed 20 % of the LLOQ level, in the retention times of internal samples did not exceed 5 % of the peak area* | | | | | | | | | | | | | | | |
| LLOQ | | 50 ng/g | | 50 ng/g | | 1.25 µg/g | | 75 ng/g | | 150 ng/g | | 200 ng/g | | 80 ng/g | | 67.5 ng/g | |
| Calibration range (linear dependence) | | 50-1200 ng/g | | 50-1200 ng/g | | 1.25-30.00 µg/g | | 75-1800 ng/g | | 150-3600 ng/g | | 200-4800 ng/g | | 80-1920 ng/g | | 67.5–1620 ng/g | |
| **Accuracy and coefficient of variation** | | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** | **Acc., %** | **CV, %** |
| Series 1 ($n = 6$**) | LLOQ | 112.73 | 8.34 | 109.61 | 12.06 | 104.46 | 13.48 | 82.64 | 6.28 | 103.52 | 9.49 | 115.69 | 7.62 | 86.68 | 9.39 | 100.42 | 7.97 |
| | LQC | 92.25 | 8.66 | 96.79 | 8.94 | 95.74 | 7.96 | 88.31 | 5.01 | 98.19 | 8.97 | 97.73 | 9.49 | 98.98 | 9.11 | 102.87 | 3.34 |
| | MQC | 97.49 | 8.59 | 90.04 | 7.47 | 97.45 | 6.48 | 86.98 | 2.30 | 111.27 | 5.75 | 98.66 | 6.60 | 104.73 | 5.71 | 109.07 | 2.72 |
| | HQC | 104.51 | 3.78 | 91.97 | 4.04 | 100.89 | 5.95 | 89.17 | 2.95 | 114.60 | 3.25 | 105.36 | 3.78 | 108.76 | 7.68 | 110.87 | 3.96 |
| Series 2 ($n = 6$**) | LLQC | 85.44 | 8.06 | 94.21 | 12.11 | 90.42 | 6.40 | 110.94 | 7.00 | 108.36 | 6.91 | 114.02 | 5.72 | 101.01 | 6.36 | 111.76 | 3.39 |
| | LQC | 99.82 | 9.09 | 92.70 | 9.06 | 95.90 | 10.04 | 93.18 | 3.59 | 106.26 | 3.19 | 106.73 | 5.69 | 110.72 | 1.40 | 102.01 | 3.73 |
| | MQC | 96.97 | 9.16 | 91.98 | 8.04 | 92.33 | 8.77 | 87.29 | 3.36 | 113.84 | 4.22 | 97.66 | 5.03 | 104.49 | 6.52 | 96.82 | 2.55 |
| | HQC | 104.38 | 9.21 | 101.75 | 6.78 | 96.28 | 6.17 | 93.33 | 2.72 | 111.91 | 2.13 | 99.15 | 3.12 | 112.17 | 5.00 | 102.51 | 3.01 |
| Series 3 ($n = 6$**) | LLQC | 101.48 | 13.05 | 98.56 | 12.01 | 106.51 | 5.59 | 92.11 | 3.81 | 116.19 | 4.06 | 112.96 | 4.18 | 100.72 | 11.90 | 117.89 | 2.41 |
| | LQC | 93.43 | 9.26 | 93.27 | 7.95 | 92.70 | 4.95 | 91.44 | 3.09 | 110.03 | 6.76 | 108.07 | 3.10 | 100.96 | 8.29 | 109.49 | 3.73 |
| | MQC | 89.70 | 3.36 | 96.11 | 5.02 | 91.00 | 2.70 | 93.15 | 3.48 | 95.31 | 10.62 | 98.65 | 3.44 | 99.25 | 1.80 | 105.30 | 2.93 |
| | HQC | 87.67 | 5.39 | 99.14 | 5.51 | 93.76 | 7.27 | 96.03 | 3.22 | 86.07 | 2.35 | 97.61 | 3.20 | 105.11 | 3.46 | 106.65 | 2.61 |
| Accuracy and coefficient of variation between series ($n = 18$**) | LLQC | 99.89 | 15.58 | 100.79 | 14.08 | 100.46 | 12.13 | 92.11 | 3.81 | 109.36 | 8.70 | 114.22 | 6.29 | 102.55 | 12.13 | 109.99 | 8.42 |
| | LQC | 95.17 | 9.95 | 94.26 | 9.14 | 94.78 | 8.37 | 91.44 | 3.09 | 104.82 | 8.36 | 104.17 | 8.01 | 110.45 | 8.75 | 104.80 | 4.95 |
| | MQC | 94.72 | 8.78 | 92.71 | 7.65 | 93.59 | 7.37 | 93.15 | 3.48 | 106.81 | 10.67 | 98.32 | 5.36 | 102.82 | 5.91 | 103.73 | 5.82 |
| | HQC | 98.85 | 10.72 | 97.62 | 7.26 | 96.98 | 7.35 | 96.03 | 3.22 | 103.31 | 13.00 | 100.70 | 4.90 | 108.68 | 6.49 | 106.68 | 4.71 |
| Dilution effect ($n = 6$) | Acc., % | 93.77 | | 90.73 | | 94.99 | | 94.71 | | 89.76 | | 93.58 | | 91.17 | | 93.36 | |
| | CV, % | 4.63 | | 4.05 | | 3.60 | | 5.88 | | 2.77 | | 3.97 | | 4.68 | | 4.24 | |

**Note.** * – selectivity evaluation was performed within series 1; ** – number of samples at each concentration level; Acc. (accuracy) – deviation of the mean value of calculated concentrations from the nominal value.

**TABLE 7**

**RESULTS OF STABILITY ASSESSMENT OF ANALYTES IN BRAIN HOMOGENATES**

| Indicators | | | Adr | NA | Dop | 5HT | 5HIAA | DOPAC | HVA | VMA |
|---|---|---|---|---|---|---|---|---|---|---|
| % of initial concentration | STS (24 hours at room temperature) (*n* = 6*) | LQC | 100.88 | 96.58 | 99.85 | 91.80 | 96.93 | 97.80 | 99.01 | 101.79 |
| | | HQC | 100.47 | 94.03 | 97.51 | 90.64 | 105.85 | 102.21 | 103.88 | 107.47 |
| | FTS (*n* = 6*) (3 cycles) | LQC | 100.60 | 101.04 | 101.40 | 95.56 | 97.26 | 100.69 | 102.62 | 105.00 |
| | | HQC | 103.22 | 96.78 | 103.04 | 97.63 | 101.48 | 101.13 | 102.01 | 107.44 |
| | ASS (48 h at +4 °C) (*n* = 6*) | LQC | 102.81 | 98.42 | 98.76 | 96.21 | 99.61 | 100.65 | 101.47 | 100.41 |
| | | HQC | 102.48 | 95.96 | 96.22 | 93.82 | 100.83 | 97.95 | 100.79 | 104.58 |
| | LTS (30 days at a temperature not exceeding -20 °C) (*n* = 6*) | LQC | 103.11 | 100.12 | 104.84 | 95.50 | 98.41 | 102.41 | 102.43 | 96.99 |
| | | HQC | 102.25 | 99.08 | 100.74 | 98.70 | 98.75 | 97.84 | 98.65 | 98.85 |

**Note.** * – number of samples at each concentration level; FTS – freezing/thawing stability after 3 cycles; LTS – long-term stability.

**TABLE 8**

**RESULTS OF QUANTIFICATION OF NORADRENALINE, ADRENALINE, DOPAMINE, SEROTONIN, DOPAC, 5HIAA, VMA, AND HVA IN RAT STRIATUM SAMPLES**

| | Concentration, ng/g | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Adr | NA | Dop | 5HT | 5HIAA | DOPAC | HVA | VMA |
| Mean values (*n* = 6) | less than LLOQ | 354.85 | 9181.30 | 595.86 | 1180.49 | 1851.35 | 896.26 | less than LLOQ |
| SD | – | 33.43 | 1497.84 | 137.25 | 244.61 | 283.25 | 266.38 | – |
| CV, % | – | 9.42 | 16.31 | 23.03 | 20.72 | 15.30 | 29.72 | – |

## DISCUSSION

Following the selection of optimal analysis conditions for the preparation of brain tissue samples after their mechanical homogenisation, sample dilution with methanol solution of internal standards was chosen. This significantly improves sample preparation throughput compared to work that has used liquid-liquid extraction [18], dialysis [13, 17, 19], and analyte derivatisation [13, 14]. The use of HPLC-MS/MS provides an advantage in selectivity and sensitivity of the method over HPLC-UV [8] and HPLC-EM [9-12] in performing pharmacodynamic studies of drugs. For instance, when using these methods, the molecules of MPs under study may be extracted and coeluted together with the analytes, thus giving overestimated quantification results [3]. The chromatographic analysis time is 21 min, which is longer than in the methods of E. Grouzmann et al. [5], N. Hwang et al. [6], L. Fang et al. [7], S. Greco et al. [14], C. Ji et al. [15]. These studies, however, analyse a smaller number of monoamine neurotransmitters and their metabolites.

A stabiliser selection approach was adopted by pre-assessing the short-term stability of analytes in homogenates and the stability of samples in the autosampler both without and with added antioxidants. A similar approach was used for blood plasma samples in [21], but it did not involve ASS testing. Reasonable results with the choice of antioxidant were achieved only when aqueous solutions of ascorbic acid in a volume ratio of 1:5 were added to the homogenate supernatant (Tables 5, 7). This is due to the fact that in methanol samples, salt reducing agents ($Na_2SO_3$, $Na_2S_2O_3$, $Na_2S_2O_5$) dissolve much worse than the selected stabilizer. In the study of long-term stability, a temperature of -20 °C or less is considered to be sufficient for sample storage (Table 7).

The concentrations of Dop, NA, 5HT, 5HIAA, DOPAC, and HVA in rat striatum samples obtained in the course of method validation coincide with the data published in J. Lu et al. [2] and N.N. Khlebnikova et al. [10]. No Adr and VMA chromatographic peaks were revealed in the chromatograms of the tested samples, indicating that the samples were collected correctly.

## CONCLUSION

The developed method for quantitative determination of norepinephrine, adrenaline, dopamine, serotonin, DOPAC, 5HIAA, VMA, HVA in rat brain samples conforms to the requirements of NCESMP, EAC, ICH, FDA guidelines in terms of selectivity, calibration dependence, accuracy and coefficient of variation within and between cycles, dilution effect, carry-over effect from previous sample, matrix effect, stability. The implementation of the chosen stabilisation method prevents oxidation of analytes during sample preparation and analysis, as well as their storage for at least 30 days. It substantially reduces the risks of unreliable results in preclinical pharmacodynamic testing and exclusion of pharmacologically effective compounds from the experiment as a consequence of falsely underestimated concentrations of neurotransmitters and their metabolites in the samples.

**Study limitations**

This method has been validated and shown to be convenient for the determination of analytes in rat brain tissue samples. The use of the method for quantification of the substances under study in another animal species would require partial validation by assessing the matrix effect, calibration dependence and selectivity.

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**Conflict of interest**

The authors declare no conflict of interest.

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## PHTHISIOLOGY

# ORGANIZATIONAL ASPECTS OF MEDICAL REHABILITATION OF PATIENTS WITH RESPIRATORY TUBERCULOSIS

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## ABSTRACT

***Background.*** *Despite the visible successes of domestic phthisiology at the present time, the problem of medical rehabilitation of patients with tuberculosis remains relevant. In recent years, approaches to medical rehabilitation of patients with various diseases have changed significantly, which entail the need to consider a complex of rehabilitation measures for patients with tuberculosis from the perspective of the Procedures for organizing medical rehabilitation in adults and children and their integration into phthisiatric practice.*

***The aim of the study.*** *To study the organizational aspects of medical rehabilitation of patients with respiratory tuberculosis in the world and the Russian Federation.*

***Materials and methods.*** *We carried out an analysis of domestic and foreign literature, regulatory documents on the organization of rehabilitation for tuberculosis patients for 2018–2023 in electronic databases PubMed/Medline, Google Scholar using terms "tuberculosis, pulmonary/rehabilitation" in English and Russian languages.*

***The results*** *show a growing amount of factual information demonstrating the positive effect of pulmonary rehabilitation in patients with respiratory diseases, including tuberculosis. The analysis revealed defects in the organization of the medical rehabilitation system in the structure of medical care for tuberculosis patients in the Russian Federation. This concerns problems of routing, phasing, organizational models, human and material resources, standardization of the main components of the rehabilitation process, the significance and effectiveness of certain rehabilitation measures, which leads to low availability of rehabilitation care for tuberculosis patients. Deficiencies in the regulatory framework prevent the integration of medical rehabilitation into the practice of TB services.*

***Conclusion.*** *Modern issues of organizing rehabilitation care for patients with tuberculosis require further study and improvement. The development of a system of medical rehabilitation of patients with tuberculosis helps to increase the effectiveness of treatment, to reduce the number of complications, disability, mortality due to tuberculosis, and to increase the duration and quality of life of patients.*

***Key words:*** *tuberculosis, post-tuberculosis pulmonary disease, rehabilitation, pulmonary rehabilitation, physical and rehabilitation medicine*

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# ОРГАНИЗАЦИОННЫЕ АСПЕКТЫ МЕДИЦИНСКОЙ РЕАБИЛИТАЦИИ БОЛЬНЫХ ТУБЕРКУЛЁЗОМ ОРГАНОВ ДЫХАНИЯ

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## РЕЗЮМЕ

***Обоснование.*** *Несмотря на видимые успехи отечественной фтизиатрии в настоящее время, проблема медицинской реабилитации больных туберкулёзом остаётся актуальной. В последние годы значительно изменились подходы к медицинской реабилитации пациентов при различных заболеваниях, которые влекут за собой необходимость рассмотрения комплекса реабилитационных мероприятий у больных туберкулёзом с позиций Порядков организации медицинской реабилитации взрослых и детей и интеграцию их во фтизиатрическую практику.*

***Цель исследования.*** *Изучить организационные аспекты медицинской реабилитации больных туберкулёзом органов дыхания в мире и Российской Федерации.*

***Методы.*** *Выполнен анализ отечественной и зарубежной литературы, нормативно-правовых документов по вопросам организации реабилитационной помощи больным туберкулёзом за период 2018–2023 гг. в электронных базах PubMed/Medline, Google Scholar по терминам «tuberculosis, pulmonary/rehabilitation», «туберкулёз/лёгочная реабилитация».*

***Результаты*** *показывают растущий объём фактических данных, свидетельствующих о положительном эффекте лёгочной реабилитации у пациентов с респираторной патологией, в том числе при туберкулёзе. Проведённый анализ выявил дефекты организации системы медицинской реабилитации в структуре медицинской помощи больным туберкулёзом в Российской Федерации. Это касается вопросов маршрутизации, этапности, организационных моделей, кадровых и материальных ресурсов, стандартизации основных составляющих реабилитационного процесса, значимости и эффективности тех или иных реабилитационных мероприятий, что ведёт к низкой доступности реабилитационной помощи для больных туберкулёзом. Недостатки нормативно-правовой базы препятствуют встраиванию медицинской реабилитации в практику фтизиатрической службы.*

***Заключение.*** *Современные вопросы организации реабилитационной помощи больным туберкулёзом требуют дальнейшего изучения и совершенствования. Развитие системы медицинской реабилитации больных туберкулёзом способствует повышению эффективности лечения, снижению количества осложнений, инвалидизации, смертности по причине туберкулёза, увеличению продолжительности и качества жизни больных.*

***Ключевые слова:*** *туберкулёз, посттуберкулёзное лёгочное заболевание, реабилитация, пульмореабилитация, физическая и реабилитационная медицина*

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## INTRODUCTION

In the early 1990s, the World Health Organization (WHO) designated tuberculosis as a global health problem. Since then, efforts to confront the disease have increased and the statistics of tuberculosis cases have decreased [1].

The epidemic situation with TB in the Russian Federation (RF) in 2022 remains stable. The tuberculosis incidence rate remains at 31.0 per 100,000 population. The TB mortality rate in 2021 was 4.3 per 100,000 population. The prevalence of tuberculosis in the civilian population fell to a historic low (58.5 per 100,000 population). The prevalence of tuberculosis with bacterial excretion is 26.6 %; among patients discharging bacteria, 56.9 % are patients with multidrug-resistant tuberculosis. More than a quarter (26.1 %) of TB patients have concomitant HIV infection [2].

I.A. Vasilieva et al. revealed that «despite a significant decrease in the TB incidence rate in 2020, in 2021, the decline continued simultaneously with a heavier clinical structure of tuberculosis: an increase in the proportion of newly diagnosed tuberculosis patients with pulmonary tissue destruction, massive bacterial excretion and fibrotic cavernous tuberculosis' [3], which plays one of the leading roles in the causes of TB-related disability in Russia [4].

Thanks to new and constantly improving diagnostic and treatment methods, there are currently about 155 million individuals successfully treated from TB worldwide [5, 6]. However, both TB itself and chemotherapy can lead to irreversible effects in the body. Tuberculosis patients report a transition from an acute state to a life with multiple chronic conditions affecting post-tuberculosis pulmonary changes, neurological impairments, cardiac and psychiatric disorders leading to poor quality of life and increased risk of death [7-9].

Pulmonary rehabilitation is an important component of the recovery of patients with chronic lung disease. The official statement of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) defines pulmonary rehabilitation as «a comprehensive intervention based on a thorough patient assessment, followed by individualised therapy, including but not limited to exercise, education and behaviour change, designed to improve the physical and psychological well-being of people with chronic respiratory disease and to promote long-term adherence» [10].

There is strong evidence that pulmonary rehabilitation improves health status, physical performance, social functioning and is recommended in international guidelines [11]. Much of the evidence supporting the benefits of pulmonary rehabilitation comes from studies in groups of patients with chronic lung disease, predominantly chronic obstructive pulmonary disease and bronchiectatic disease [12]. There is emerging evidence on post-tuberculosis pulmonary disease rehabilitation, but more data are required to determine its effectiveness [13].

Despite the visible successes demonstrated by domestic phthisiatry currently, the problem of medical rehabilitation (MR) of tuberculosis patients remains highly relevant. Over recent years, approaches to the implementation of MR in patients with various diseases have changed significantly, which entails the need to consider the complex of rehabilitation measures in tuberculosis patients from the perspective of the Procedures for Organising Medical Rehabilitation of Adults and Children, approved by the orders of the Ministry of Health of Russia No. 788n dated July 31, 2020 and No. 878n dated October 23, 2019 [14, 15], and their integration into phthisiatric practice. Therefore, in this article, we would like to address the problems of MR in TB patients, considering the new concept of the development of a comprehensive rehabilitation system in the Russian Federation.

## THE AIM OF THE STUDY

To study the organizational aspects of medical rehabilitation of patients with respiratory tuberculosis in the world and the Russian Federation.

## MATERIALS AND METHODS

The analysis of domestic and foreign literature, regulatory and legal documents related to the organisation of comprehensive rehabilitation care for tuberculosis patients over the period 2018–2023 in the electronic databases PubMed/Medline, Google Scholar by the terms «tuberculosis, pulmonary/rehabilitation», «tuberculosis/pulmonary rehabilitation» was performed.

## RESULTS

Rehabilitation is an integral part of the health care system along with health promotion, disease prevention, treatment and palliative care. As health systems have grown in many countries around the world, the survival rate of patients after serious illness and severe injury has increased, but the number of people with residual complex functional impairment leading to disability has also increased. According to WHO estimates, about 2.4 billion people in the world currently suffer from diseases for which rehabilitation is indicated [16]. The prevalence of disability in most European countries is about 10 %, which leads to a certain burden of care for both individuals and society as a whole, increasing the costs of medical and social care [17].

Rehabilitation is an effective way to reduce disability as well as increase opportunities for people with impaired function. The structure, funding and accessibility of rehabilitation services vary from state to state and depend on health systems. In order to create common basic principles for the organisation of rehabilitation care, the European Union of Medical Specialists (EUMS) has published

a White Paper on Physical and Rehabilitation Medicine (PRM), which outlines its main positions in Europe, defining the specialisation, functioning, competencies and professional qualities of PRM specialists, based on advanced standards of care in accordance with the evidence base and in the context of various national recommendations and practices [17].

A new definition of «medical rehabilitation» is introduced by the Federal Law «About the Fundamentals of Citizens` Health Protection in the Russian Federation» No. 323-FZ dated November 21, 2011 [18]. Follow-up care and rehabilitation of tuberculosis patients in the Russian Federation are guaranteed by Federal Law No. 77-FZ dated June 18, 2001 «About prevention of tuberculosis spread in the Russian Federation» [19]. The procedure for follow-up care of tuberculosis patients, persons who are or have been in contact with the source of tuberculosis, as well as persons suspected of tuberculosis and those cured of tuberculosis, regulates the examination of patients, treatment, and medical rehabilitation of these persons. Follow-up care is implemented by TB specialists on the basis of clinical recommendations and in accordance with the standards of medical care, whose functions include rehabilitation measures in addition to dispensary appointments (examinations, consultations) [20].

Procedures for the organisation of medical rehabilitation of adults and children [14, 15] also prescribe adherence to clinical recommendations and standards of care as one of the basic conditions for MR activities. Tuberculosis clinical guidelines for both adult and children's tuberculosis state that rehabilitation of patients with tuberculosis should start from the very beginning of the patient's treatment and is mainly limited to the use of movement regimen and high-protein diet as pathogenetic treatment methods aimed at restoring the patients' health. In addition, rehabilitation measures include other drug and non-drug components of pathogenetic treatment, the main objective of which is to restore specific and non-specific reactivity of the patient's organism. Psychological and/or social support to build adherence to treatment is also categorized as rehabilitative [21, 22].

The above-mentioned clinical guidelines for patients with tuberculosis receiving treatment in the continuation phase, in the absence of contraindications, recommend the sanatorium phase of treatment [21, 22]. The Procedure for the provision of medical care to patients with tuberculosis defines the «Rules for organising the activities of a sanatorium for the treatment of tuberculosis of all forms», in which MR of persons placed on the TB dispensary register is declared as one of the main functions of a phthisiatric sanatorium [23].

The procedures for organizing medical rehabilitation for both adults and children specify a stage of MR implementation. The first stage of MR is recommended to be implemented in structural subdivisions of a medical organisation providing specialised, including high-tech, medical care in an *inpatient facility under the profile 'TB'*, where rehabilitation measures should be initiated in the acute (up to 72 hours) and peracute periods of the disease course in emergency conditions, conditions after surgical interventions (in the early postoperative period), chronic critical conditions and should be carried out daily for at least 1 hour, but not more than 3 hours.

The second stage of MR is implemented at the *inpatient medical rehabilitation departments created in the health care organisations*, including medical rehabilitation centres, sanatorium-resort organisations (SRIs) [14, 15]. However, the current Procedure for the Provision of Medical Care to Patients with Tuberculosis does not provide for the organisation of medical rehabilitation departments in the structure of TB institutions; accordingly, there are no staffing and equipment standards [23]. Nowadays, in fact, the second stage of MR of tuberculosis patients is partially implemented in tuberculosis sanatoria as part of the provision of sanatorium-resort care. MR activities in the second stage should be conducted in the early recovery period of the disease course and during the residual effects of the disease course and should be performed daily for at least 3 hours [14, 15].

The third stage of MR is implemented when providing primary medical and sanitary care in an outpatient basis and (or) in a day hospital (*outpatient medical rehabilitation department, medical rehabilitation department of a day hospital*), including in MR centres, SRIs. MR activities in the third stage are implemented at least once every 48 hours, lasting at least 3 hours [14, 15].

Sanatorium-resort treatment has been and remains an important link in the MR of TB patients at the second and third stages of TB treatment. The preamble of one of the current orders of the Ministry of Health of the Russian Federation regulating the organisation of sanatorium-resort care for patients in tuberculosis sanatoria states that «the use of natural and pre-formed therapeutic factors, kumiss therapy, therapeutic nutrition and active motor regimen makes it possible to increase the effectiveness of treatment and accelerate the rehabilitation process» [24]. The Russian Federation has preserved a network of TB sanatoria, which have vast scientific and practical experience and potential in the rehabilitation of TB patients [25-27].

Unfortunately, the role of rehabilitation measures and sanatorium-resort treatment as a tool to improve the effectiveness of TB patients' treatment from the perspective of evidence-based medicine is currently underestimated. According to the latest available official statistics from 2019, there is a reduction in the number of sanatoria and the number of sanatorium beds in the Russian Federation for adults and children diagnosed with tuberculosis, where only 3.2 % of newly diagnosed patients with tuberculosis and 7.0 % of the contingents on dispensary registration at the end of the year were hospitalised, which indicates the low availability of rehabilitation measures for patients in the second and third stages of MR [28]. For this reason, the position of G.S. Balasanyants, who believes that the modern concept of organising sanatorium treatment should provide for the expansion of the role and importance of tuberculosis sanatoriums, which is determined by the principles of the national phthisiatric doctrine, has not lost its significance [29].

Indications towards the organizational model that allows solving the tasks of providing comprehensive medical, social and rehabilitation care at the third stage of MR are presented on the example of an outpatient phthisiatric institution of St. Petersburg «TB Dispensary No. 5», where a department of medical and social care and rehabilitation was formed. N.V. Korneva et al. point out the exclusivity of the existing department, which required the development of all regulatory documentation, including the department's regulations, functional responsibilities of employees, algorithms of work and routing, and rehabilitation programmes [30].

To determine individual patient routing when implementing MR measures at different stages, the Procedure for Organising Medical Rehabilitation proposes the Rehabilitation Routing Scale (RRS) [14].

If the RRS score is 0-1, the patient does not need rehabilitation (only secondary prophylaxis is indicated), if the score is 2-3, a course of treatment in a stage 3 MR unit (day hospital) is indicated, and if the score is 4-6, a course of treatment in a specialised stage 2 MR unit (round-the-clock unit/on-site home rehabilitation course/telemedicine consultation) is indicated (Fig. 1).

We found no sources in the available literature that assessed the status of TB patients by RRS, which is necessary to clarify the need for rehabilitation care for TB patients at all stages of MR.

Rehabilitation measures are implemented by a multidisciplinary rehabilitation team (MDRT), which is a structural and functional unit of a structural subdivision of a health care organisation or other organisation, established on a functional basis from the employees of the specified departments [14, 15]. The MDRT is led by a doctor of physical and rehabilitation medicine, a specialist who meets the requirements of the professional standard "Specialist in Medical Rehabilitation" [32].

The MDRT may include: physical and rehabilitation medicine doctor/medical rehabilitation doctor, physical rehabilitation specialist, ergorehabilitation specialist, medical psychologist/psychotherapist, medical speech therapist, medical rehabilitation nurse, ward nurse, exercise therapist, physiotherapist, reflexologist, physical therapy instructor, physical therapy instructor, physiotherapy nurse, massage nurse, reflexology nurse, physical therapy instructor [14, 15].

One can't but agree with the opinion of N.V. Korneva et al. [30], that despite the fact that one of the tasks of a TB dispensary, according to the Procedure for the Provision of Medical Care to Patients with Tuberculosis, is the implementation of rehabilitation measures for patients with tuberculosis [23] and the professional standard of a doctor-phthisiatrist contains the labour function – "conducting and monitoring the effectiveness of inpatient medical rehabilitation of tuberculosis

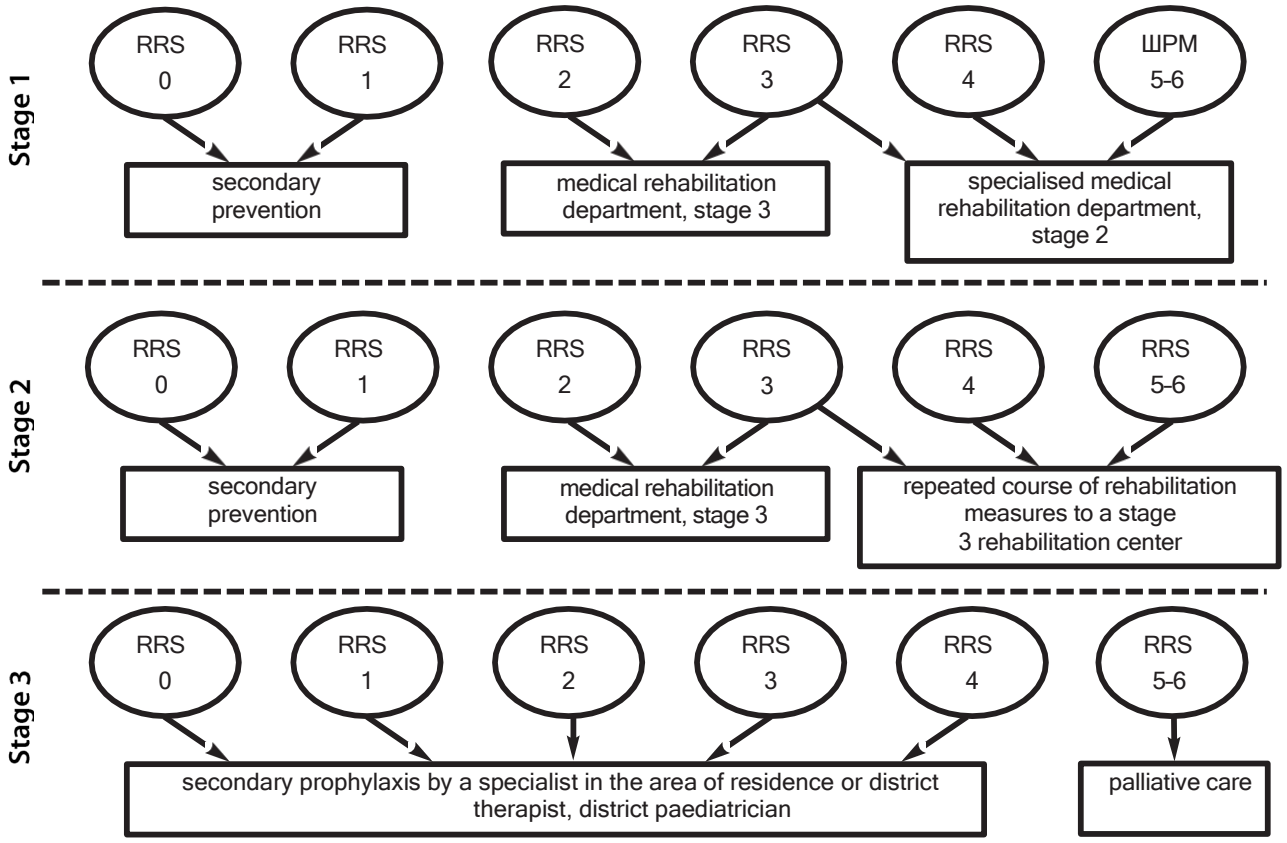


***FIG. 1.***
*Patient routing at the stages of medical rehabilitation [31]*

patients and persons with post-tuberculosis residual changes during the provision of specialised medical care, including the implementation of individual rehabilitation or habilitation programmes for disabled persons" [33], the recommended staffing standards of phthisiatric institutions, including sanatoriums providing treatment of tuberculosis of all forms, do not include specialists required for organisation and implementation of MR, in accordance with the Procedure for Organisation of Rehabilitation Care. According to Order No. 932n, TB dispensaries should have a medical and social assistance room only in the structure of an outpatient counselling department, the staff of which includes a head, a medical methodologist, a medical psychologist, a social worker, and a medical and social assistance nurse [23]. This staffing is clearly insufficient to form the MDRT.

When implementing MR activities at all stages of MDRT, a rehabilitation diagnosis is established, including the characterisation of the state of functioning and disability (function, body structure, activity and participation of the patient), the influence of environmental and personal factors based on the International Classification of Functioning, Disability and Health (ICF) and its changes in the course of MR activities [14, 15].

In 2001, the International Classification of Functioning, Disability and Health was published, belonging to the «family» of international classifications developed by WHO, applicable to various aspects of health, providing common rules for coding a wide range of health-related information, and using a standardised common language to enable communication on health and health-related issues worldwide in different disciplines and branches of science [34].

E.V. Melnikova et al. in their published instruction concerning the use of ICF in outpatient and inpatient medical rehabilitation (2017) emphasised the main aspect of rehabilitation, which before the creation of ICF was focused around disorders of functions and structures, which led to active medical care, but other, non-medical problems of the patient were not considered. ICF implementation has shifted the focus of the professional's attention to functioning rather than function, manifested by a better perception of the impairments present from the categories of activity and participation, as well as personal and environmental factors, which allows a broader view of the patient's problems in order to make a rehabilitation diagnosis, determine the goal and objectives of rehabilitation, and make better use of available resources [35].

The ICF today includes more than 1.6 thousand different categories [34], which significantly complicates its practical application, and in this regard, reduced versions of classification for specific nosological forms of diseases, the so-called basic sets of ICF, the formation of which is based on the results of scientific studies based on many thousands of samples, in which specialists of the relevant profile, related specialties, with the participation of a group of international experts of WHO [36]. There are currently more than 50 ICF core sets developed and published for the most common diseases such as stroke, ischaemic heart disease, traumatic brain injury, etc. [37]. Unfortunately, tuberculosis is not one of them.

The basic set of ICFs simplifies the development of a rehabilitation programme for a specific pathology in a specific patient, allows to make it, on the one hand, comprehensive, on the other – as individual as possible. There is a large number of works in the public domain showing the practical application of ICF for health assessment and rehabilitation measures in various diseases, resulting in the formation of effective rehabilitation programmes, the implementation of which leads to the solution of the set tasks and achievement of the MR goal of the described patients [38, 39].

In order to adequately assess the various ICF categories and identify the actual problems of the examined patient, a number of special examination methods are widely used in medical rehabilitation: laboratory and instrumental studies, various international scales, tests and questionnaires according to the pathology for objectification of which they were created. Different variations of their use in specific clinical examples were demonstrated by G.E. Ivanova et al. The authors believe that the creation of a unified tool for assessing a patient's general condition based on the ICF principles using modern methods of patient examination, clinical tests and scales accepted by the professional community will help MDRT in making a rehabilitation diagnosis and in determining a more accurate rehabilitation potential, which will ensure higher efficiency of medical rehabilitation in general [36].

In phthisiatric practice, the «Scale for assessing functional deficit in tuberculosis patients» that was developed using the ICF was proposed by T.V. Pyaznova et al. (2018); it was used to assess clinical and laboratory signs of internal organ failure, impaired communication, mobility and self-care in tuberculosis patients with HIV infection [40, 41].

In summary, the development and validation of a basic set of ICF domains in TB patients, selection of laboratory and instrumental research methods, special tests and scales to describe ICF categories, development of criteria for assessing the severity of disability, distribution of domains of the basic set of ICF domains among MDRT specialists for assessment procedures, and determination of the relationship between ICF categories and certain rehabilitation measures should become one of the directions of further studies.

One of the main principles of MR is the early initiation of rehabilitation measures, which is important in terms of preventing degenerative changes in tissues and provides a more favourable course and outcome of the disease, serves as one of the moments of disability prevention [42]. However, foreign literature sources most often cover the issues of pulmonary rehabilitation of patients after tuberculosis [13].

Studies have shown that up to 50 % of TB patients report disease-related health problems after completing treatment [43-45], which negatively affect quality of life with negative consequences for psychological, social and economic components and reduce overall life expectancy [46-48]. Post-tuberculosis pulmonary changes

in the form of bronchiectasis, bronchial stenosis, cavitation, fibrotic nodular scarring and pleural thickening lead to changes in the elasticity of lung tissue, gas exchange, functional lung volumes and airflow with consequent impairment of lung ventilation [13, 49]. A significant proportion of patients who have completed TB treatment therefore report residual cough, weakness, dyspnoea, difficulty climbing stairs or with activities of daily living, at work. The true burden of post-tuberculosis conditions is not fully known due to the lack of epidemiological data, but some authors estimate that they affect 18–87 % of patients cured of tuberculosis [50].

In 2019, the first International Symposium on Post-Tuberculosis Disease was held in Stellenbosch, South Africa, where the definition of post-tuberculosis pulmonary disease (PTPD) was adopted as features of chronic respiratory pathology with or without symptoms that can be at least partially attributed to prior (pulmonary) tuberculosis [51, 52]. There is an urgent need to acknowledge PTPD as a leading cause of chronic lung disease and to conduct more research into its diagnosis, pathophysiology, and optimal person-centred management to reduce morbidity and achieve better treatment outcomes in clinically cured TB patients [53, 54].

The International Union Against Tuberculosis and Pulmonary Disease has published Guidelines for Post-Tuberculosis Pulmonary Disease [13], based on the Global Plan to End Tuberculosis [55], and Clinical Standards for the Assessment, Management and Rehabilitation of Post-Tuberculosis Pulmonary Disease [51], which represent the first formal attempt to develop a consensus approach to this important global problem by international experts. The document contains general principles that need to be adapted to specific circumstances and situations for the subsequent implementation of rehabilitation programmes. Five standards are proposed, including: a basic set of examinations to detect PTPD, an identification of indications in patients with PTPD for pulmonary rehabilitation, a statement of the main components of the rehabilitation programme, methods for assessing the effectiveness of rehabilitation measures and a scheme for patient assessment during TB treatment and follow-up, and a summary of the components of educational programmes [51].

Preliminary data from studies in TB survivors suggest that pulmonary rehabilitation programmes may be beneficial for people with PTPD dyspnoea and pulmonary dysfunction. Access to pulmonary rehabilitation programmes in high TB burden settings is currently limited, and more data are needed to understand the best combination of tools and techniques that could be both useful and widely available to health care providers and recipients [56]. Studies examining pulmonary rehabilitation in individuals with PTPD have mainly used a holistic approach including methods such as 6-minute walk test, breath-hold tests, breathing exercises, drainage breathing techniques, nutritional advice and psychological support [13].

Studies from both high- and low-income countries suggest that pulmonary rehabilitation programmes for people with PTPD are viable and are associated with improved quality of life, physical performance and respiratory outcomes [13]. Evidence of specific pulmonary rehabilitation programmes tailored to patients with PTPD also exists in institutions with adequate resources, logistics and qualified staff; and these have generally been found to be effective [57-59].

There are reports of successful use of simplified programmes that do not require significant material inputs and equipment. The ability to modulate pulmonary rehabilitation programmes by adapting them to patient personality factors and available resources makes pulmonary rehabilitation potentially accessible to individuals (including children and adolescents) in a variety of settings [13, 51, 58].

An increasing body of evidence thus demonstrates the positive effects of pulmonary rehabilitation in patients with chronic pulmonary disease. Despite these important benefits, however, pulmonary rehabilitation is universally underutilised and referral, coverage and completion rates are alarmingly low. Worldwide, less than 3 % of patients with chronic pulmonary disease benefit from pulmonary rehabilitation [60]. One of the key impediments for pulmonary rehabilitation referrals is the poor recognition of its benefits by health care organisations, due in part to the limited resources and funding available for pulmonary rehabilitation services. The two most common barriers for patients to be referred to pulmonary rehabilitation are related to a lack of knowledge among health care providers about the content of pulmonary rehabilitation programmes and its benefits [61].

## CONCLUSION

The analysis of publications and current regulatory and legal regulatory background in the field of rehabilitation in the Russian Federation and publications in domestic and foreign sources testifies to the imperfection of the organisation with regard to the medical rehabilitation system in the structure of medical care for tuberculosis patients. This concerns issues of routing, phasing, organisational models, human and material resources, standardisation of the rehabilitation process's main components, and the significance and effectiveness of certain rehabilitation measures, which leads to low accessibility of rehabilitation care for TB patients.

Shortcomings in the legal and regulatory framework hinder the integration of MR into the practice of the phthisiatric service. To organise an effective system of rehabilitation care for tuberculosis patients, it is necessary to form rehabilitation units within the structure of TB institutions in compliance with the staffing norms for MDRT formation and equipment standards recommended by the Procedure for Organising Medical Rehabilitation, for which it is necessary to make appropriate additions to the Procedure for Providing Medical Care to Tuberculosis Patients.

Nowadays, the issues related to the organisation of rehabilitation care for tuberculosis patients require further study and improvement. It is crucial to determine the indications for rehabilitation measures, comprehensive

examination of the patient using diagnostic tools, assessment tools, scales and questionnaires to establish the rehabilitation diagnosis, and the patient's priority problems from the ICF perspective, in order to personalise pulmonary rehabilitation programmes to achieve the rehabilitation goal and effectively address the patient's individual needs.

Medical rehabilitation development as part of medical care for tuberculosis patients will ultimately contribute to improving the effectiveness of tuberculosis treatment, reducing the number of complications, disability, mortality associated with tuberculosis, and increasing the life expectancy and quality of life of patients.

**Conflict of interest**

The authors declare the absence of apparent and potential conflicts of interest related to the publication of this article.

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## EXPERIMENTAL RESEARCHES

# THE EFFECT OF MELATONIN ON THE BCL-2 AND BAD PROTEINS EXPRESSION IN OVARIAN CORPUS LUTEUM CELLS AFTER EXPOSURE TO EXPERIMENTAL HYPERTHERMIA

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## ABSTRACT

***Background.*** *There is growing interest in determining the role of melatonin in the regulation of proliferation and apoptosis of ovarian cells at various diseases and destabilizing influences. It is believed that the choice between the implementation of a cell death or survival program determines the ratio of anti-apoptotic and pro-apoptotic proteins.*
***The aim.*** *To identify the effect of melatonin on the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bad and the Bcl-2/Bad ratio in the ovarian luteocytes of Wistar rats in the acute (day 3) and recovery (days 7 and 14) periods after a single exposure to experimental hyperthermia.*
***Materials and methods.*** *Warming up took no more than 17 minutes. Melatonin was injected subcutaneously (0.1 mg in 0.2 ml of physiological solution) for 3 days after experimental hyperthermia. Comparison groups included rats with physiological solution injection (control) and animals after experimental hyperthermia + physiological solution injection. The Bad and Bcl-2 expression was determined immunohistochemically on days 3, 7 and 14 after experimental hyperthermia + physiological solution or melatonin injection.*
***Results.*** *On the day 3 after experimental hyperthermia, the effect of the hormone was not detected. A week after experimental hyperthermia + melatonin injection, the Bad expression area decreased more significantly than in rats after experimental hyperthermia + physiological solution injection, which led to an increase in Bcl-2/Bad ratio. This indicated an increase in anti-apoptotic protection, blocking the development of the internal apoptosis pathway at this time. 2 weeks after experimental hyperthermia + physiological solution injection, the Bcl-2 area decreased more significantly than the Bad area. As a result, the Bcl-2/ Bad ratio decreased almost 2-fold compared to the control group. This indicated the activation of the "mitochondrial branch" of luteocyte apoptosis. Two weeks after experimental hyperthermia + melatonin injection, the Bad and Bcl-2 areas decreased synchronously, which restored Bcl-2/Bad to control values.*
***Conclusion.*** *The melatonin injection after experimental hyperthermia shifts the ratio of Bcl-2/Bad expression areas towards an increase in anti-apoptotic Bcl-2 already a week after the recovery period and promotes earlier normalization of Bcl-2/Bad to physiological levels (as early as 2 weeks after experimental hyperthermia + melatonin injection).*

***Key words:*** *melatonin, apoptosis, experimental hyperthermia, rat ovaries, corpus luteum, luteocytes, Bad, Bcl-2, Bcl-2/Bad*

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# ВЛИЯНИЕ МЕЛАТОНИНА НА ЭКСПРЕССИЮ БЕЛКОВ BCL-2 И BAD В КЛЕТКАХ ЖЁЛТЫХ ТЕЛ ЯИЧНИКОВ ПОСЛЕ ВОЗДЕЙСТВИЯ ЭКСПЕРИМЕНТАЛЬНОЙ ГИПЕРТЕРМИИ

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## РЕЗЮМЕ

*Растёт интерес к выяснению роли мелатонина (МТ) в регуляции пролиферации и апоптоза клеток яичников при различных заболеваниях и дестабилизирующих воздействиях. Считается, что выбор между реализацией программы гибели или выживания клетки определяет соотношение антиапоптотических и проапоптотических белков.*

***Цель.*** *Выявить влияние мелатонина на экспрессию антиапоптотического Bcl-2 и проапоптотического Bad и соотношение Bcl-2/Bad в лютеоцитах яичников крыс Вистар в острый (3-и сутки) и восстановительный (7-е и 14-е сутки) периоды после однократного воздействия экспериментальной гипертермии (ЭГ).*

***Методы.*** *Разогревание составляло не более 17 минут. МТ вводили подкожно (0,1 мг в 0,2 мл физиологического раствора (ФР)) в течение 3 суток после ЭГ. Группы сравнения – крысы с введением ФР (контроль) и животные после ЭГ и ФР. Экспрессию Bad и Bcl-2 определяли иммуногистохимически на 3-и, 7-е и 14-е сутки после ЭГ и введения МТ/ФР.*

***Результаты.*** *На 3-и сутки после ЭГ эффект гормона не выявлялся. Через неделю после ЭГ + МТ площадь экспрессии Bad уменьшалась значительнее, чем у крыс после ЭГ + ФР, что приводило к росту Bcl-2/Bad. Это свидетельствовало об увеличении антиапоптотической защиты, блокирующей развитие внутреннего пути апоптоза на данном сроке. Через 2 недели после ЭГ + ФР площадь Bcl-2 уменьшалась значительнее, чем площадь Bad. В результате Bcl-2/Bad практически в 2 раза снижался по сравнению с контролем. Это свидетельствовало об активации «митохондриальной ветви» апоптоза лютеоцитов. Через 2 недели после ЭГ + МТ площади Bad и Bcl-2 уменьшались синхронно, что восстанавливало Bcl-2/Bad до контроля.*

***Заключение.*** *Введение МТ после ЭГ сдвигает соотношение площадей экспрессии Bcl-2/Bad в сторону увеличения антиапоптотического Bcl-2 уже через неделю восстановительного периода и способствует более ранней нормализации Bcl-2/Bad до физиологического уровня (уже через 2 недели после ЭГ + МТ).*

***Ключевые слова:*** *мелатонин, апоптоз, экспериментальная гипертермия, яичники крыс, жёлтые тела, лютеоциты, Bad, Bcl-2, Bcl-2/Bad*

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## INTRODUCTION

In modern conditions, the impact of high temperature to the human body is increasing as a result of global climate change, the development of regions with hot climate, the development of tourism and migration, as well as work in a number of industries (metallurgical, coal, mining, etc.). Meanwhile, to date, extensive scientific material has been accumulated about the use of hyperthermia for the therapy of oncological, infectious, parasitic diseases, drug-dependent conditions, AIDS. General hyperthermia in the temperature range from 42.5° to 44.0 °C is an extreme factor of the environment, to the action of which the body responds with a combination of complex changes that lead to profound disorders of cellular and extracellular relationships in biological structures and significant disorders of blood circulation and lymph flow, the development of hypoxia and stimulation of apoptosis. The reproductive organs, in particular the ovaries, which are the centre of the female reproductive system, are under considerable strain in these conditions [1].

Crucially, stress-induced regulated cell death also represents a strategy for preserving biological equilibrium, resembling an adaptive response to stress.

Two main pathways of apoptosis signal transduction are distinguished: receptor-dependent (external) signalling pathway involving cell death receptors expressed on the cell membrane surface, and mitochondrial (internal or intrinsic) pathway. The intrinsic apoptosis pathway is a form of regulated cell death triggered by various changes in the microenvironment, including DNA damage, endoplasmic reticulum stress, excess reactive oxygen species, etc. The mitochondrial signalling pathway of apoptosis is activated as a result of increased permeability of the mitochondrial outer membrane, release of apoptogenic proteins from the mitochondrial intermembrane space into the cell cytoplasm and subsequent triggering of a whole cascade of reactions leading to the development of programmed cell death. An important role in the mechanisms of regulation of programmed cell death is assigned to the inhibitor of apoptosis – Bcl-2 protein, which prevents translocation of Bcl-2-associated cell death promoters Bax and Bad by oligomerisation with these proteins and thus blocks the release of proapoptotic small molecules from mitochondria [2]. The ratio of active forms of apoptosis inhibitors and inducers determines the choice between the implementation of the cell death programme and cell survival and is informative in determining the degree of apoptosis inhibition [3]. The ability to influence the interaction between anti-apoptotic and pro-apoptotic members of the BCL-2 family through either pharmacological or genetic interventions is of great importance in medicine for the treatment of various (cancer, autoimmune, neurodegenerative, etc.) diseases [3].

Any stresses and extreme exposures, including hyperthermia, lead to disturbances in the body's detoxification and adaptation systems. The epiphysis hormone melatonin (MT) is involved in restoring disturbed homeostasis and optimising the functions of various organs and systems. This hormone, which has a wide range of properties, is the main synchronizer of the body's endogenous rhythms, as well as a powerful antioxidant. Moreover, MT is an antioxidant that is found in the mitochondria of cells, and at higher concentrations than in other organelles or subcellular sites. It is believed that MT can even be synthesized in mitochondria [4]. The ability to regulate cell proliferation and apoptosis is one of the most significant physiological properties of epiphyseal MT [5, 6].

Epiphyseal MT is involved in the regulation of sex hormone secretion and puberty processes, thus ensuring the full functioning of the reproductive system. MT deficiency in mice leads to follicle atresia and accelerates age-related fertility decline [7]. This hormone is a key regulator of human reproductive functions [8].

The corpus luteum serves as a temporarily functioning organ and defines a crucial role in the regulation of the estrous cycle and maintenance of pregnancy. The luteal function is largely performed by progesterone, the main steroid hormone synthesised by this gland. MT has been found to play a key role in reproductive physiology by regulating the production of prolactin, follicle-stimulating and luteinising hormones. MT synthesis in the ovaries and testes reflects the auto- and paracrine regulation of reproductive physiology, ensuring high quality ova and sperm. The hormone is produced in the cells of the epithelium, stroma, and myometrium and is involved in maintaining the homeostasis of the organ by regulating multiple pathways associated with the processes of decidualisation and implantation [9]. MT functions as an important regulator in the ovary, as indicated by the expression of melatonin receptors MT1 and MT2 in different compartments of the ovary [10]. The presence of MT1 and MT2 in luteocytes and the regulatory role of MT in the endocrine function of the ovarian luteal bodies of horses [11], pigs, and mice [10, 12] have been confirmed. It has been revealed that this hormone is able to increase progesterone release by corpus luteum in gestating sows [10], and to induce progesterone production by granulosa and luteal cells in humans [13]. MT activates a set of genes expressed in the sows' and mice corpus luteum associated with progesterone synthesis, including cytochrome P450 family 11 subfamily A member 1 (*Cyp11a1*), aldo-keto reductase family 1, member C18 (*Akr1c18*), isopentenyl diphosphate delta isomerase 1 (*Idi1*) and luteinising hormone/choriogonadotropin receptor (LHCGR), and consequently increases progesterone production in sows [14].

There is now increased interest in elucidating the role of MT in the regulation of cell proliferation and apoptosis in a number of different ovarian cells. The aspects concerning the role of MT and its effect on the «mitochondrial branch» of apoptosis in luteocytes of ovarian luteal bodies in experimental models of overheating are, however, insufficiently covered in scientific publications. This determined our interest in studying the effect of MT

on programmed cell death processes in ovarian luteal cells when exposed to high temperature.

## THE AIM OF THE STUDY

To analyse the effect of melatonin after a single exposure to experimental hyperthermia (EH) against the expression of apoptosis inhibitor Bcl-2 and inducer of programmed cell death protein Bad and against the Bcl-2/Bad ratio in luteocytes of Wistar rats in acute (3rd day) and recovery (7th and 14th day) periods.

## MATERIALS AND METHODS

The study was conducted on 3-month-old female Wistar rats with body weight 180-200 g. The animals were kept in a certified vivarium of the Central Research Laboratory of the Novosibirsk State Medical University of the Ministry of Health of Russia at an air temperature of 20–22 °C on a standard dietary intake and with free access to water. Experiments were performed in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union governing the protection of animals used for scientific purposes and the rules of good laboratory practice. The study was approved by the Ethical Committee of the Research Institute of Clinical and Experimental Lymphology – branch of the Federal Research Centre of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Minutes No. 128 dated March 15, 2017). Sexual cycle phases were determined using the vaginal swab method [15]. Rats in the diestrus phase of the sexual cycle were exposed once to EH in accordance with the «Method of experimental modelling of general hyperthermia in small laboratory animals» [16]. According to the method of modelling general hyperthermia, animals were warmed up in the tank of a standard TB-110 thermobath when immersed in hot water up to the neck level. The thermobath design provides for automatic maintenance of water heating temperature and uniform mixing of its layers, that allows to consider the temperature of the coolant as a constant value in the experiment. The advantage of modelling general hyperthermia in an aqueous environment over an air environment is that uniform, deep and rapid heating of the animal body is achieved. The heating temperature regime of hot water – warm carrier was selected experimentally and equalled 45 °C. The time of warming up of each individual to the level of rectal temperature of 43.5 °C (heat shock stage) was no more than 17 minutes and was individual. No deaths of rats from hyperthermia have been reported.

Three groups were formed: 1st (control) – rats without EH exposure, which were subcutaneously injected with 0.2 ml of physiological solution (PS); 2nd (EH + PS) – animals exposed to EH and received 0.2 ml of PS; 3rd (EH + MT) – animals exposed to EH and received MT. MT (ICN Biomedicals Inc., USA) was administered subcutaneously at a dose of 0.1 mg in 0.2 ml of PS. The first MT injection was administered on the day of EH, then – in the following 2 days (in the evening, after sunset, once a day). Animals were removed from the experiment under ether anaesthesia 3 (acute period), 7 and 14 days (recovery period) after exposure to EH and MT, that corresponded to the ideas of phasicity in the course of the posthyperthermic period. There were 5 individuals from each group for each point of withdrawal from the experiment.

Ovaries were fixed in 10 % neutral formalin solution, then dehydrated in a series of alcohols of increasing concentration and encapsulated in paraffin. Immunohistochemical study of Bcl-2 and Bad protein expression was performed on 3 μm thick paraffin sections of ovaries by indirect two-step streptavidin-biotin method using Novostain 500 kit (NCL-RTU-D, Novocastra, UK), mouse monoclonal antibodies to anti-apoptotic protein Bcl-2 (IgG, No. 610538; BD Biosciences, USA) and to the proapoptotic protein Bad (IgG, No. 610392; BD Biosciences, USA). In the last step, immunohistochemical staining was performed in a chromogenic substrate containing diaminobenzidine. Sections were examined and microphotographs were obtained using an AxioImager M2 motorised microscope (Carl Zeiss, Germany) with an AxioCam HRc camera (Carl Zeiss, Germany) at a final magnification of 630x. Quantitative assessment over the relative areas of stained sections was performed using the computer programme Axio Vision 4.7.1 (Carl Zeiss, Germany) and an automatic measurement unit (NEXIV AutoMeasure; Nikon, Japan). Bcl-2/Bad area ratios were calculated.

Statistical processing of the obtained data was performed in Statistica 6.1 program (StatSoft Inc.; serial number AXXR101E832903FA). As a baseline for each marker, the differences in mean visual field within a group were compared. Each sample included up to 50 fields of view per group for each marker. The samples corresponded to a normal distribution. The values of arithmetic mean and standard error of the mean were calculated. Statistical significance of the differences between the compared values was determined using the parametric Student's criterion. The differences were considered statistically significant at $p < 0.05$. Median, first and third quartile values were determined for the Bcl-2/Bad ratio. Statistical significance of the differences between the compared values was evaluated using the nonparametric Mann – Whitney U-criterion. Differences were considered statistically significant at $p < 0,05$.

## RESULTS

In corpus luteum cells on the 3rd day of the experiment, when both placebo and MT were administered after EH, the expression areas of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bad increased synchronously (Fig. 2). Along with this, there was an increase in the intensity of luteocyte staining for both proteins (Fig. 1). Consequently, in animals of both groups, the Bcl-2/Bad ratio remained at the control level (Fig. 3). This is an evidence

that the intensity of apoptosis in ovarian luteal cells during the acute period after EH remains within the physiological values, and at this stage MT has no statistically significant effect on the development of cell death.

One week after EH and PS administration, the areas of Bcl-2 and Bad expression in luteocytes and the Bcl-2/Bad ratio were persisted at 3 days (Fig. 2, 3). MT administration after hyperthermia contributed to a decrease in staining intensity (Fig. 1, 2) and decreased values for both proteins compared to all groups (Fig. 2). The expression area of the pro-apoptotic protein Bad, however, decreased more significantly than that of the anti-apoptotic protein Bcl-2 (Fig. 2). In consequence, the Bcl-2/Bad ratio was statistically significantly increased at this recovery period compared to all groups (Fig. 3). This is an evidence of earlier establishment of anti-apoptotic defence blocking the development of the intrinsic pathway of apoptosis of ovarian luteal cells already on the 7th day after hyperthermia and MT administration compared to animals without MT treatment.

On the 14th day of the experiment in rats receiving PS after EH, the area of Bcl-2 expression in ovarian luteal cells decreased, more significantly than for Bad (Fig. 2).

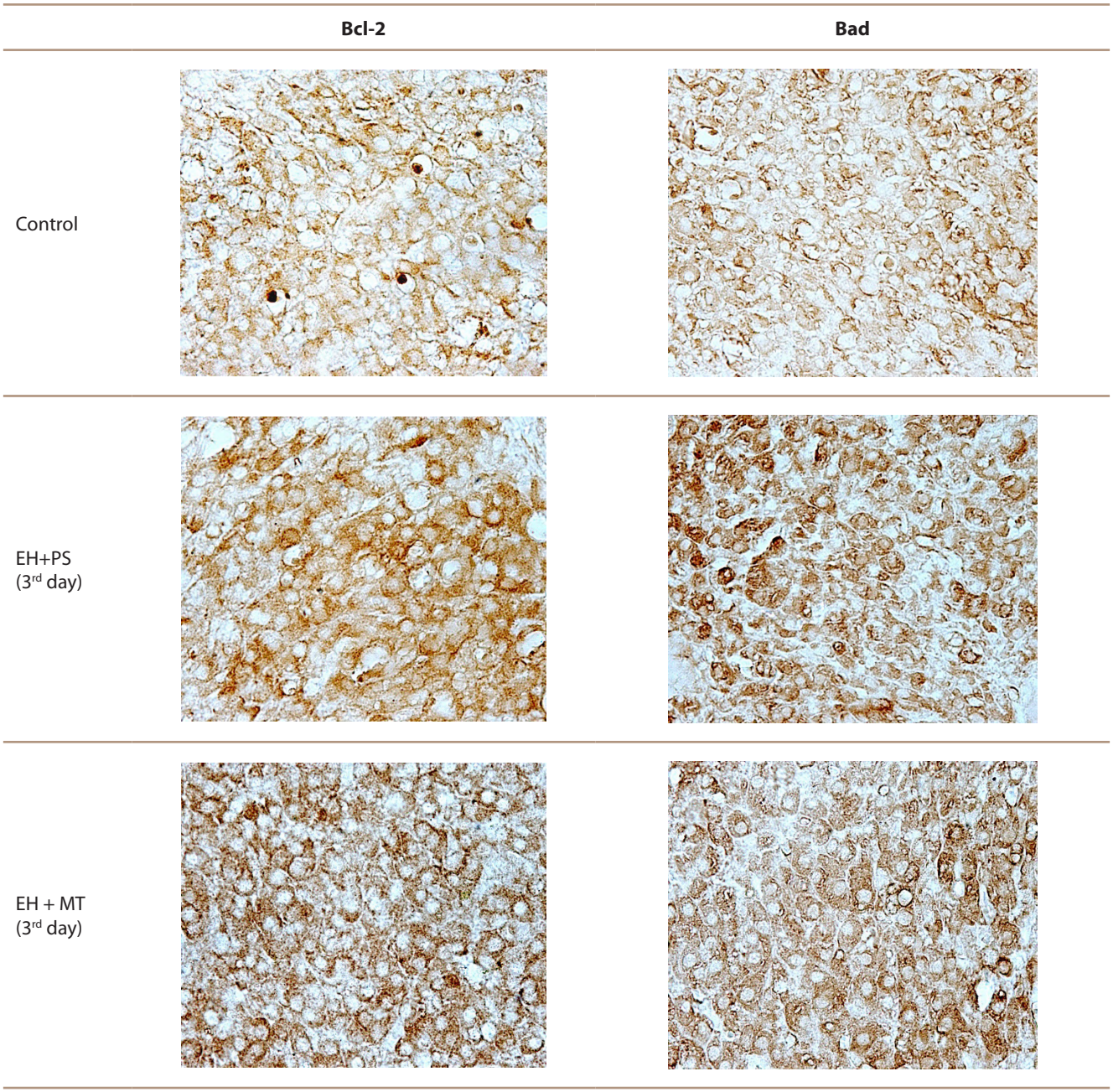
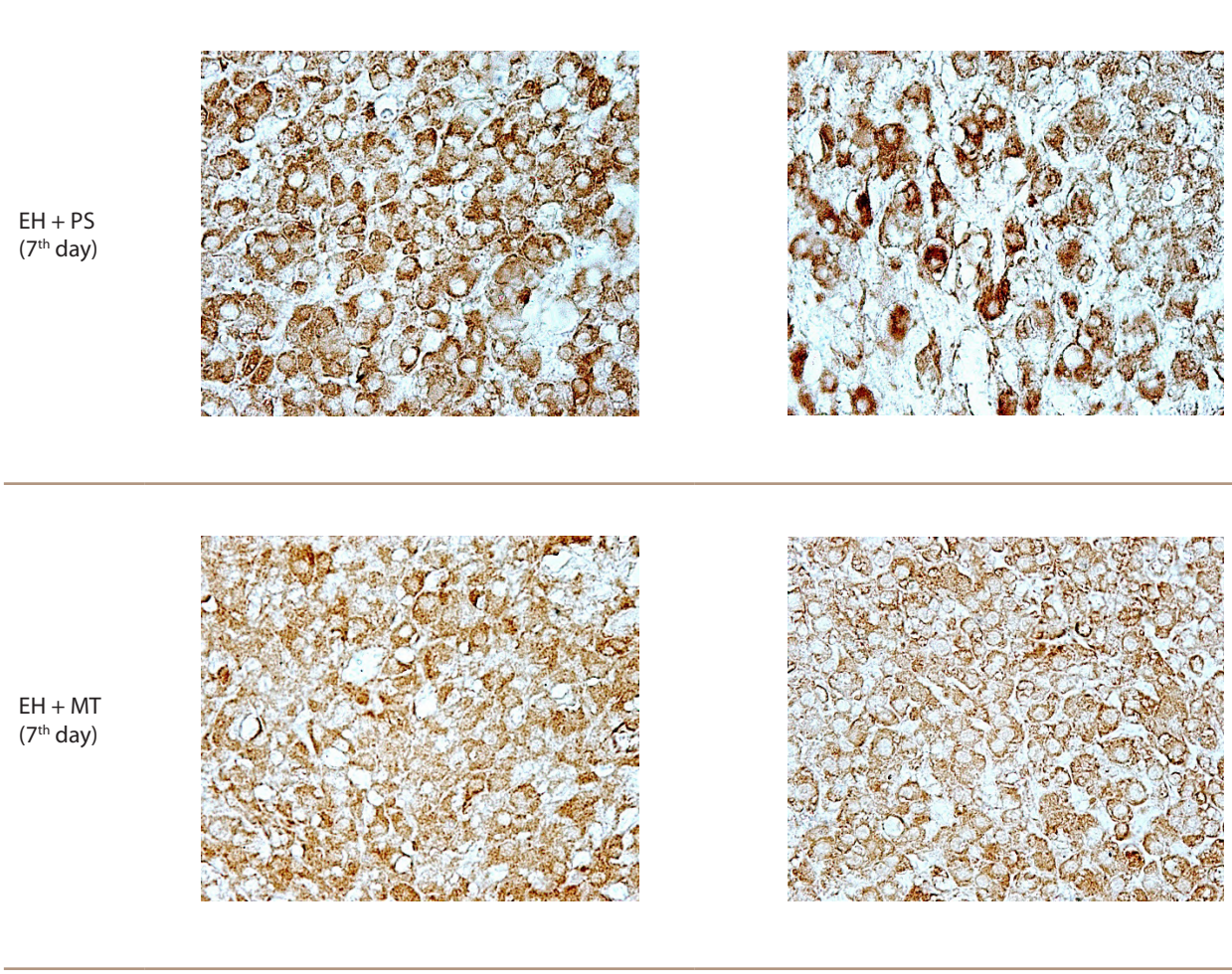
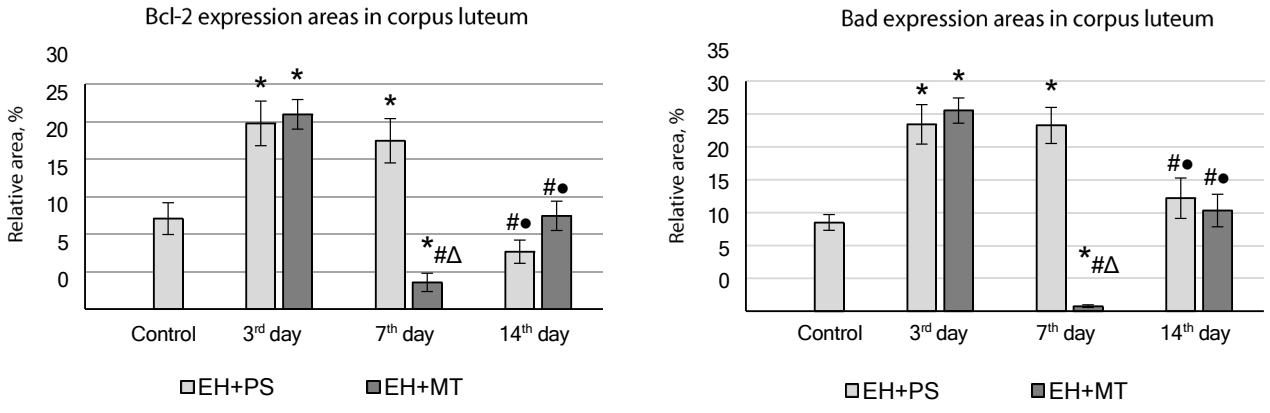



***FIG. 1.***
*Microphotographs of rat ovarian corpora lutea on the 3rd day after exposure to experimental hyperthermia (EH) and administration of physiological saline (PS) or melatonin (MT). Immunohistochemical staining by indirect streptavidin-biotin method for anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bad in corpus luteum cells; magnification × 400*




*a*




*b*

***FIG. 2.***
*a – microphotographs of rat ovarian corpora lutea on the 7th day after exposure to EH+PS and EH+MT; immunohistochemical staining by indirect streptavidin-biotin method for Bcl-2 and Bad in the cells of corpora lutea; magnification × 400. б – graphs of the expression areas of the studied proteins in different terms of the experiment ($p < 0.05$): * – compared to control; # – compared to 3rd day; ● – compared to 7th day; Δ – intergroup comparison of the same term*

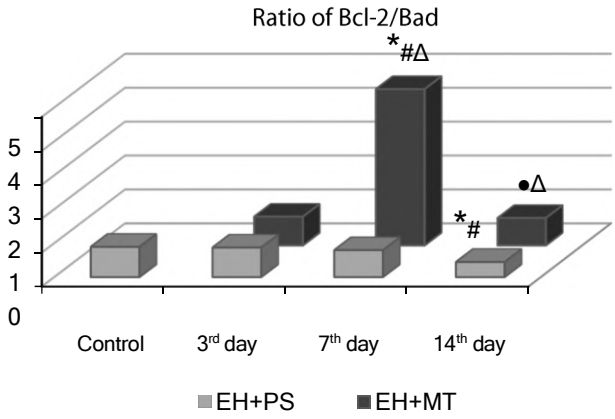



***FIG. 3.***
*Ratio of Bcl-2/Bad expression areas at different experimental time intervals ($p < 0.05$): * – compared to control; # – compared to 3rd day; ● – compared to 7th day; Δ – intergroup comparison of the same time period*

As a result, the Bcl-2/Bad area ratio decreased almost 2-fold compared to the control (Fig. 3). It evidences the activation of the «mitochondrial branch» of programmed cell death of luteocytes at this stage of the recovery period. MT administration to hyperthermia-treated animals promoted a decrease in staining intensity (Fig. 4) and a more pronounced decrease in Bad compared to the decrease in Bcl-2, leading to the restoration of the Bcl-2/Bad index to control levels (Fig. 2, 3). The obtained results indicate the cytoprotective effect of MT administration to animals after hyperthermia, which as early as on the 14th day of the recovery period ensures the development of mitochondrial pathway of luteocyte apoptosis within the physiological norm.

## DISCUSSION

Heat stress is a known promoter of the reactive oxygen species (ROS) formation that can jeopardise pregnancy and fetal development. Overheating / thermal stress has deleterious effects against oocyte development potential in pigs, mice, and cattle, including adverse effects

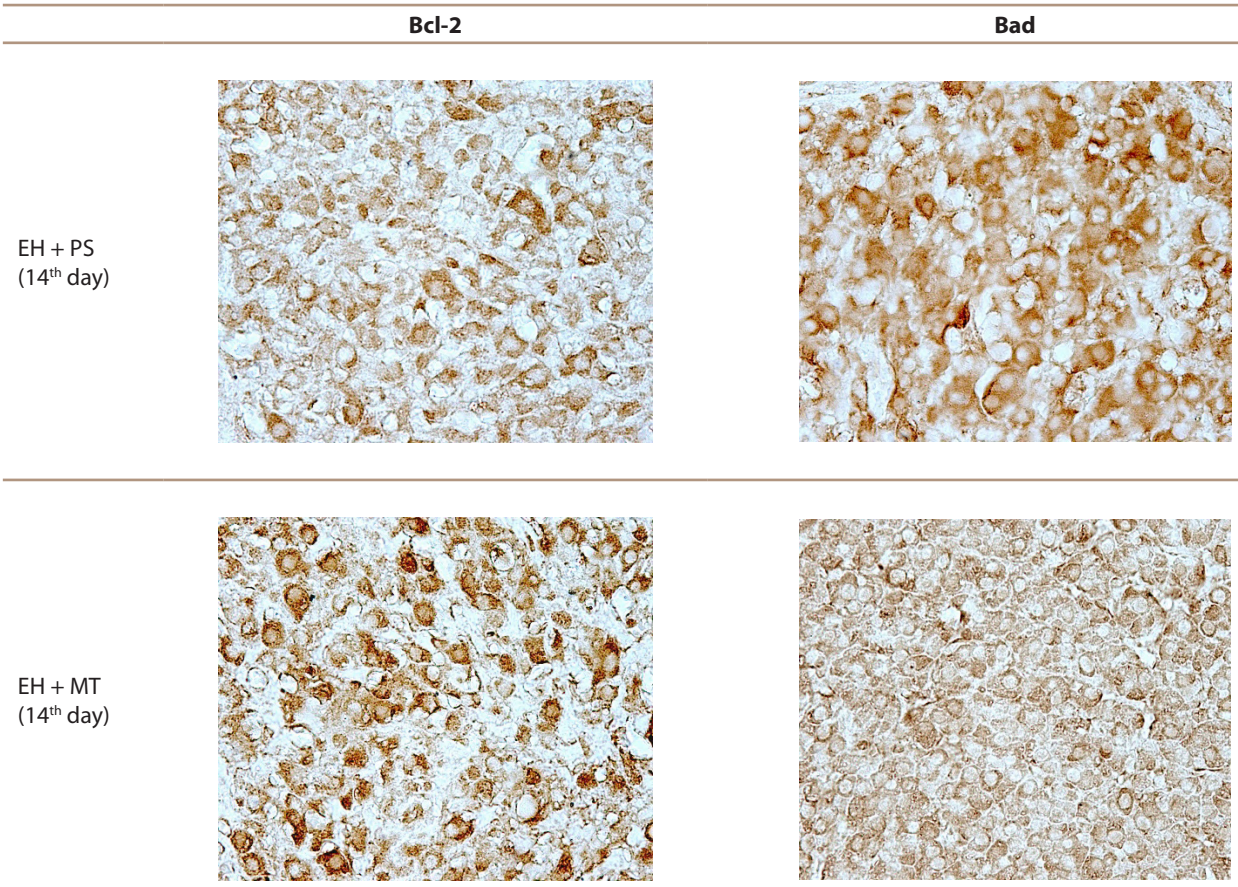



***FIG. 4.***
*Microphotographs of rat ovarian corpora lutea on the 14th day after exposure to EH+PS and EH+MT. Immunohistochemical staining by indirect streptavidin-biotin method for Bcl-2 and Bad in ovarian luteocytes; magnification × 400*

against survivability, maturation rate, and meiotic competence of oocytes, the formation of F-actin and α-tubulin in them, and oocyte expression of the NRF2, CDK1, and GDF9 genes [17].

MT is a pleiotropic molecule that regulates various processes including pregnancy. MT usage under heat stress in pigs improves the quality and rate of oocyte maturation (partly by restoring the distribution of F-actin) [17], and has a positive effect on the duration of the estrous period and embryo survival [18]. The use of MT reduces ROS production in maturing cattle oocytes and increases the ability to develop embryos from heat shocked oocytes [17]. MT administration throughout pregnancy in heat stressed sheep improves their redox status and leads to an increase in the average number of offspring, lamb weight, and milk production [19]. Under heat stress, this hormone was observed to suppress p53 expression and increase luteinising hormone level and *Bcl-2* gene expression in sheep granulosa cells [20].

Our studies revealed that the effect of high external temperature led to a change in the ratio of apoptosis regulator proteins (predominance of pro-apoptotic protein Bad over anti-apoptotic protein Bcl-2) in ovarian luteal 2 weeks after EH. Of significance, the expression area of the anti-apoptotic protein Bcl-2 decreased significantly more than the expression area of the pro-apoptotic protein Bad, resulting in a 2-fold decrease in the Bcl-2/Bad ratio index compared to control. It evidenced the insufficiency of anti-apoptotic protection and activation of the «mitochondrial branch» of luteocyte apoptosis. Exposure to hyperthermia promotes to the occurrence of state of oxidative stress. The damaging effects of such exposures lead to mitochondrial dysfunction and excessive accumulation of ROS in them, that promotes cell apoptosis in various ovary compartments. In consequence, due to its antioxidant and anti-apoptotic effects, MT can significantly reduce oxidative stress and restrain apoptosis [20-22]. At the same time, the ratio of active forms of pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-xL) proteins will determine the choice between the implementation of the cell survival or cell death programme [3]. Specifically, Bcl-2 can prevent translocation of apoptosis inducers Bax and Bad by oligomerising with these proteins and thereby block the release of these molecules from mitochondria, that in turn will restrain apoptosis. Bcl-2 has been found to bind more strongly to Bad than to Bax [2].

MT has been demonstrated to significantly inhibit apoptosis of thecal cells in sheep reducing the expression of the pro-apoptotic protein Bax and increasing the expression of the anti-apoptotic protein Bcl-2, that has implications for delaying ovarian atresia and aging [23]. MT has been found to inhibit the mitochondrial apoptosis pathway of granulosa cells in ovaries of cyclophosphamide chemotherapy-treated mice – reducing the increased expression levels of cleaved caspase 3, Bax, cytoplasmic Cyt-c and increasing the decreased Bcl-2 expression in ovaries [24]. MT can protect mouse ovaries from premature deficiency caused by trypterygium glycosides by reducing apoptotic damage – the hormone reduces caspase 3, Bax expression and increases Bcl-2 expression [25].

As a consequence of the present study, we found that MT administration to rats after hyperthermia shifts the ratio of Bcl-2/Bad expression areas in ovarian corpora lutea towards an increase in anti-apoptotic Bcl-2 as early as on the 7$^{th}$ day of the recovery period, in contrast to animals not treated with this hormone, which contributes to the normalisation of Bcl-2/Bad to physiological levels (as early as on the 14th day). In an earlier study, we also revealed that MT could inhibit the mitochondrial apoptosis pathway in rat ovarian follicles as early as day 7 post-hyperthermia [5].

MT, by reducing oxidative stress and apoptotic damage, protects corpus luteum cells in the ovaries. The hormone restores the number of corpora lutea and follicle density in mice and rats reduced by toxic effects [25, 26], and improves luteinising hormone levels, corpus luteum function and survival of sheep and goat embryos [19]. MT protects the corpus luteum from ROS and plays a key role in sustaining its function in women [19]. Treatment with this hormone of infertile women with luteal phase defect increases intrafollicular MT concentration, reduces intrafollicular oxidative damage, improves progesterone production by the corpus luteum and increases fertilisation and pregnancy levels [27].

MT and its MT1 receptor play an important role in luteinization. Melatonin/MT1 signalling was revealed to markedly improve the expression of corpus luteum marker genes. High-throughput sequencing results revealed that interaction with the extracellular matrix receptor, focal adhesion and activation of the PI3K/Akt pathway, which are involved in luteinisation of granulosa cells, can mediate the effects of melatonin/MT1 signalling [28]. MT increases progesterone production in the corpus luteum of gestating sows and increases the expression of both P450scc and StAR, resulting in increased luteal cell viability. Epiphyseal hormone exerts its regulatory role in luteocyte function through signalling pathways mediated by the MT1 and MT2 melatonin receptors, the presence of which has been confirmed in corpus luteum cells [10, 12].

MT-mediated signalling mechanisms through receptors for this hormone are very complex and vary depending on the type and kind of ovary cells. They mainly include the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, the phosphatidylinositol-3-kinase/apoptosis signal-regulated kinase (PI3K/AKT) pathway, and the calcium-regulated pathway [23, 29]. More specifically, MT activates the PI3K/Akt pathway, mediating progesterone synthesis and secretion by thecal cells. In addition to its antioxidant properties, MT can activate the SIRT1/PGC-1α pathway, which promotes mitochondrial biogenesis disrupted by environmental toxins [20].

Counteraction to oxidative stress and reprogramming of impaired metabolism in cells is provided by MT synthesised in mitochondria, where its distribution is much

higher than in other subcellular organelles and has no circadian rhythm. MT has been confirmed to be released from mitochondria and then controls cytochrome *c* release via the MT1 receptor on the membrane, i.e. provides automitocrine regulation. MT is present in mitochondrial membranes and is transported into mitochondria by the membrane-bound oligopeptide transporters PEPT1 and PEPT2. The high concentrations of MT and its multiple antioxidant actions provide a powerful defence for these organelles exposed to free radicals. Within mitochondria, MT acts as a direct scavenger of free radicals and associated non-radical products and stimulates antioxidant enzymes including superoxide dismutase 2, catalase and glutathione reductase, while inhibiting pro-oxidant enzymes. MT, by regulating lipoxygenase activity, protects cells from hydroperoxidation of polyunsaturated fatty acids. It modulates endoplasmic reticulum responses to stress, sirtuin activity, mitophagy and autophagy processes. MT by direct capture of ROS in mitochondria, activation of antioxidant defence and preservation of membrane integrity plays a crucial role in maintaining normal mitochondrial functions and energy metabolism in cells [9]. In summary, melatonin exposure under conditions of hyperthermia leads to a decrease in apoptotic death of corpus luteum cells and, as a consequence, to a reduction in the damaging effect of overheating on the morphological organisation of the organ.

## CONCLUSION

MT administration after EH shifts the Bcl-2/Bad expression area ratio towards an increase in the anti-apoptotic Bcl-2 protein as the 7$^{th}$ day of the recovery period. Administration of this hormone after hyperthermia promotes earlier normalisation of Bcl-2/Bad to physiological level – already on the 14$^{th}$ day after EH and MT exposure.

**Conflict of interest**

The authors declare no conflict of interest.

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# EFFECT OF SUBCHRONIC EXPOSURE TO MANGANESE ON MINERAL METABOLISM IN WISTAR RATS

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## ABSTRACT

***Background.*** *The presence of increased manganese concentrations in soils and water intakes areas for drinking and household purposes in a number of the Russian Federation subjects indicates the chronic influence of this metal on all segments of the population. This situation is aggravated by violation of the optimal nutrition principles, which leads to changes in the organism absorption of micronutrients. In this regard, the study of the elemental status of an organism against the background of manganese exposure is of particular importance.*

***The aim of the study.*** *To study the effects of subchronic exposure to manganese on the mineral status of Wistar rats.*

***Materials and methods.*** *To conduct the study, 20 mature rats were selected, from which two groups were formed – control (n = 10) and experimental (n = 10). Animals in the control group received a general diet, animals in the experimental group received a diet with additional administration of manganese sulfate at a dose of 1433 mg/kg for 28 days. At the end of the preparatory period, blood and brain samples were taken to determine the content of chemical elements using inductively coupled plasma mass spectrometry and of metal-ligand manganese forms using high-performance liquid chromatography combined with inductively coupled plasma mass spectrometry.*

***Results.*** *It has been established that subchronic oral exposure to manganese leads to an increase in the content of this microelement in the blood serum and to a decrease in the levels of calcium, potassium, magnesium, iron and copper. In the cerebral cortex, the level of manganese, lead, mercury and strontium increases against the background of a decrease in iron and iodine levels. An increase in the gross content of manganese in blood serum leads to an overload of the main high-molecular carriers and initiates the formation of low-molecular forms of manganese.*

***Conclusion.*** *Subchronic oral exposure to manganese leads to the accumulation of this microelement in the body of animals and to the development of an imbalance of a number of macro- and microelements.*

***Key words:*** *mineral metabolism, manganese, microelements, analysis of the content of chemical forms of elements, toxicity*

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# ВЛИЯНИЕ СУБХРОНИЧЕСКОГО ВОЗДЕЙСТВИЯ МАРГАНЦА НА МИНЕРАЛЬНЫЙ ОБМЕН КРЫС ЛИНИИ WISTAR

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## РЕЗЮМЕ

***Обоснование.*** *Наличие повышенных концентраций марганца в почвах, на водозаборах питьевого и хозяйственно-бытового назначения в ряде субъектов Российской Федерации свидетельствует о хроническом влиянии данного металла на все слои населения. Усугубляет данное положение нарушение принципов оптимального питания, что приводит к изменению усвоения микронутриентов организмом. В связи с этим особое значение приобретает изучение элементного статуса организма на фоне воздействия марганца.*

***Цель исследования.*** *Изучить эффекты субхронического воздействия марганца на минеральный статус крыс линии Wistar.*

***Материалы и методы.*** *Для проведения исследования было отобрано 20 половозрелых крыс, из которых были сформированы две группы – контрольная (n = 10) и опытная (n = 10). Животные контрольной группы получали общий рацион, животные опытной группы – рацион с дополнительным введением сульфата марганца в дозе 1433 мг/кг в течение 28 дней. По окончании подготовительного периода осуществлялся забор крови и головного мозга для определения содержания химических элементов методом масс-спектрометрии с индуктивно связанной плазмой и металл-лигандных форм марганца методом высокоэффективной жидкостной хроматографии в сочетании с масс-спектрометрией с индуктивно-связанной плазмой.*

***Результаты.*** *Установлено, что субхроническое пероральное воздействие марганца приводит к повышению содержания данного микроэлемента в сыворотке крови, снижению уровня кальция, калия, магния, железа и меди. В коре головного мозга повышается уровень марганца, свинца, ртути и стронция на фоне снижения железа и йода. Повышение валового содержания марганца в сыворотке крови приводит к перегрузке основных высокомолекулярных носителей и инициирует образование низкомолекулярных форм марганца.*

***Заключение.*** *Субхроническое пероральное воздействие марганца приводит к кумуляции данного микроэлемента в организме животных и развитию дисбаланса ряда макро- и микроэлементов.*

***Ключевые слова:*** *минеральный обмен, марганец, микроэлементы, анализ содержания химических форм элементов, токсичность*

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## INTRODUCTION

The vital activity of humans and animals has been substantiated to be closely related to the chemical composition of the environment and the content of various macro- and microelements, which take part in the formation of a number of adaptive mechanisms of the organism, including the functioning of vital systems, such as nervous, respiratory, cardiovascular, immune, digestive, endocrine [1].

One of the essential chemical elements associated with the geochemical parameters of the area is manganese. On the one hand, this metal is a vital trace element that controls a number of physiological processes in human and animal organisms [2]. Manganese is known to be involved in the regulation of carbohydrate, protein and lipid metabolism, protection of cells from oxidative stress, synthesis of neurotransmitters, and ensuring full reproductive function [3-5]. At the same time, at increased exogenous intake, manganese can have a negative effect on the functional state of the organism [6, 7]. This was first observed among miners, welders, smelters and other workers who were exposed to inhalation exposure to manganese as a result of the use of this metal in production cycles [8, 9]. However, it should be recognised that health risks are not limited to occupational exposures alone. The presence of manganese elevated concentrations in soils and water in a number of constituent entities of the Russian Federation indicates its chronic influence on the whole population [10]. This is especially true for the part of the population that lives near industrial areas and roads [11]. This situation is aggravated by the violation of the principles of optimal nutrition, which leads to changes in the absorption of chemical micronutrients by the body [12]. Consequently, it is food and drinking water that are the main intermediate link in the intake of manganese into the human and animal organism from the environment, largely determining the state of mineral metabolism [13]. Considering the above, the importance of studying the metabolism of macro- and microelements against the background of chronic exposure to manganese increases.

## THE AIM OF THE STUDY

To evaluate the state of mineral metabolism of Wistar rats against the background of subchronic exposure to manganese.

## MATERIALS AND METHODS

The experimental study was performed in full compliance with the ethical standards prescribed in the World Medical Association Declaration of Helsinki (1964, revised 2013). The design of the experiment was approved by the local ethical committee of the Federal Research Centre of Biological Systems and Agrotechnologies of the Russian Academy of Sciences (Orenburg). The study was performed using 20 sexually mature female Wistar rats. The age of the animals was 3 months at the time the experiment commenced. All animals received a basic diet (BD) in the form of a loose compound feed and were kept in plastic cages with sawdust bedding under conditions of artificial light (12-hour daylight hours) and supply and exhaust ventilation. The duration of the experiment was 4 weeks (28 days).

The animals were divided into two groups. Rats from the control group ($n = 10$) received the basic diet, animals of the experimental group ($n = 10$) – BD supplemented with pentahydrate manganese sulphate ($MnSO_4 \times 5H_2O$) at a dose of 1433 mg/kg. This salt is produced by JSC «Vekton» (St. Petersburg) with certified purity not less than 98 %, is a crystalline powder of white to pale pink colour (TS (technical specifications) 6-09-01-218-84). Dose selection of $MnSO_4 \times 5H_2O$ was based both on the GESTIS Substance Database and the European Chemicals Agency (ECHA) information systems.

After 28 days, the animals were removed from the experiment for the collection of biomaterials (blood, cerebral cortex) to evaluate the effect of manganese over mineral metabolism.

Blood was sampled from the cardiac artery into 6 ml VACUETTE vacuum tubes with clotting activator and gel (Greiner Bio-One International AG, Austria). Centrifugation of tubes for 10 minutes at 1000 g using ELMI CM-6M centrifuge (Latvia) was performed to obtain serum. The separated serum was stored at a temperature of -70 °C in Eppendorf-type test tubes. Immediately after rapid extraction of the brain, a tissue slice was performed from the surface of the hemisphere with a thickness of about 1–1.5 mm. Samples were weighed and stored at -20 °C until analyzed.

The content of macro- and microelements in blood serum and cerebral cortex samples was analysed using a NexION 300D mass spectrometer (PerkinElmer Inc., USA) by inductively coupled plasma mass spectrometry (ICP-MS). Determination of individual chemical forms of manganese in blood serum was performed using a NexION 300D mass spectrometer (PerkinElmer Inc., USA) in combination with a PerkinElmer Series 200 liquid chromatograph (PerkinElmer Inc., USA) by high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS). This stage of the study was conducted in the certified laboratory of Micronutrients LLC (Moscow; licence registration number L041-01137-77/00370156).

The data obtained were processed by variation statistics methods using Statistica 10 statistical package (StatSoft Inc., USA). A hypothesis that the data belong to the normal distribution was rejected in all cases with a probability of 95 %, which justified the use of non-parametric procedures for processing statistical populations (Mann – Whitney *U*-criterion). The findings are presented as median (Me) and 25$^{th}$ and 75$^{th}$ percentiles ($Q_{25}$–$Q_{75}$). The relationships between the parameters were evaluated using Spearman's Rank Correlation Coefficient. A correlation coefficient ($r$) was calculated to determine the closeness of the relationship between the studied features. Correlation coefficients were evaluated as follows:

less than 0.3 – weak relationship; 0.3 to 0.5 – moderate; 0.5 to 0.7 – significant; 0.7 to 0.9 – strong; more than 0.9 – very strong [14].

## RESULTS

The results of the study revealed that subchronic oral exposure to manganese did not lead to changes in the external signs of the animals and their activity level. However, a comparative analysis of the macro- and microelements content in blood serum revealed that in the experimental group by the end of the experiment the level of manganese statistically significantly exceeded the control values by 70 %; a tendency to increase the content of iodine and selenium was also observed. Against this background, it was revealed that the serum level of potassium was statistically significantly lower than in the control by 25 %, copper by 24 %, magnesium by 19 %, iron by 10 %, calcium by 8 %; there was a tendency to decrease phosphorus and zinc (Fig. 1).

Increased sensitivity to toxic factors of phylo- and ontogenetically younger and higher located parts of the brain compared to older and lower located parts is a general pattern for the central nervous system, in this connection we also analysed the elemental composition of the cerebral cortex [15] (Fig. 2).

It was revealed that in the grey matter of animals from the experimental group the content of a number of heavy metals was statistically significantly higher than the control values: the level of manganese exceeded the control by 19 %, strontium – by 57 %, mercury

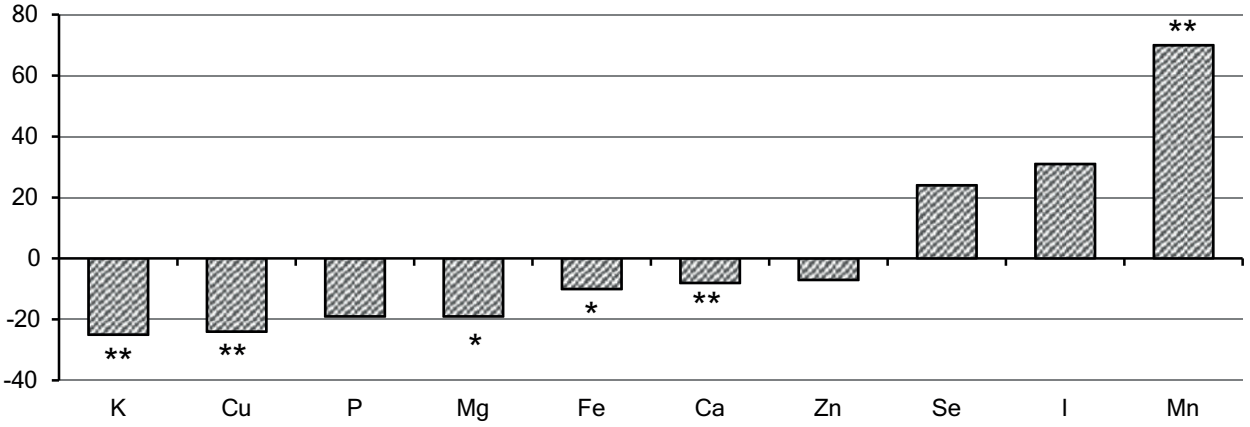


***FIG. 1.***
*Relative values of chemical elements content in blood serum of animals from the experimental group (%): X axis (0) – level of elements in the control group; * – statistically significant difference between the experimental group and control at p ≤ 0.05; ** – statistically significant difference between the experimental group and control at p ≤ 0.01*

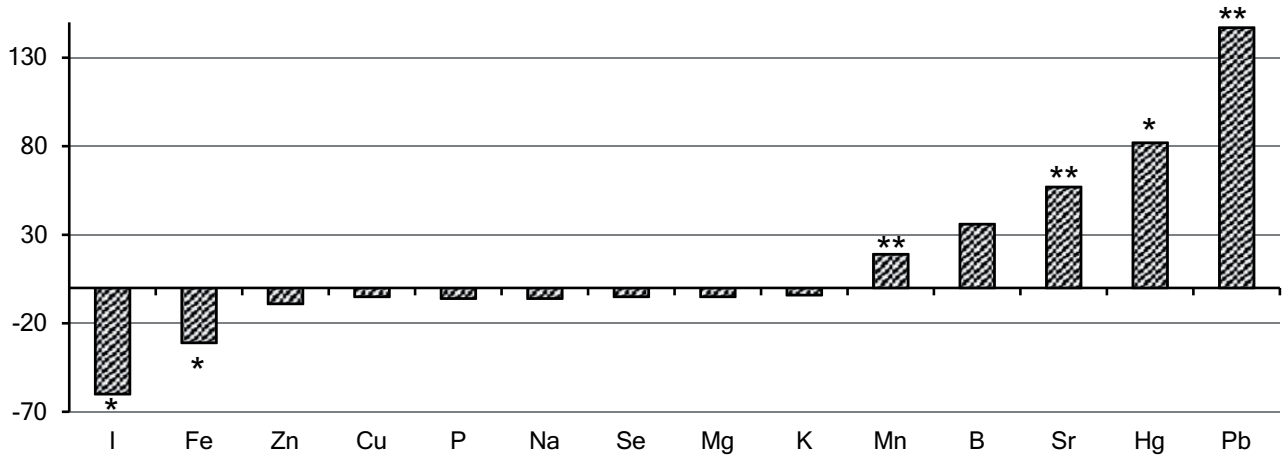


***FIG. 2.***
*Relative values of chemical elements content in the cerebral cortex of animals from the experimental group (%): X axis (0) – level of elements in the control group; * – statistically significant difference between the experimental group and control at p ≤ 0.05; ** – statistically significant difference between the experimental group and control at p ≤ 0.01*

– by 82 %, lead – by 147 %. In contrast, iron and iodine levels were statistically significantly lower than the control, by 31 % and 60 %, respectively.

From a diagnostic point of view, it is of particular interest to identify possible relationships between serum and cortical chemical elements, as the latter do not have equivalent *in vivo* measurements. Correlation matrix analysis showed that serum magnesium level was statistically significantly positively associated with cortical magnesium ($r \geq 0.3$), while serum copper level was positively correlated with brain copper ($r \geq 0.3$).

Manganese is known to be a rather active chemical element, as a result of which in the body it is stabilised in complex with various biological ligands. We have therefore analysed the chemical forms of this metal (speciation analysis). It was revealed that serum manganese of Wistar rats is mainly associated with high molecular weight compounds – such fractions as transferrin/albumin (Mn-Tf/Alb), in smaller amounts – with α-2-macroglobulin fraction (Mn-A2M); with low molecular weight compounds (Mn-LMM), and is also represented by free inorganic form (Mn-free) (Fig. 3).

It was revealed that the main amount of manganese in the experimental group of animals was presented in the form of Mn-free fraction (6.6 μg/l), exceeding the control values by 1.8 times. Among the organic forms, it was observed that the concentrations of Mn-LMM and Mn-Tf/Alb were statistically significantly higher than the control, by a factor of 4 and 1.9, respectively. A tendency to decrease the content of the Mn-A2M fraction was observed against this background.

The results of the analysis concerning the percentage distribution of Mn by fractions against the background of its different content in the blood serum of animals are presented in Figure 4 for illustrative purposes.

According to correlation analysis, it was revealed that serum gross manganese content was statistically significantly positively associated with such fractions as Mn-free ($r = 0.89$), Mn-Tf/Alb ($r = 0.81$) and Mn-LMM ($r = 0.63$); a negative correlation with Mn-A2 ($r = -0.6$) was observed (Fig. 5).

The scatter graph illustrates the fact that the higher was the level of total manganese in serum among the animals of the experimental group, the higher was the concentration of Mn-free, Mn-LMM and Mn-Tf/Alb fractions and the lower became the concentration of Mn-A2 fraction. The graph also clearly illustrates that an increase in the total manganese concentration in rat serum (above 12 μg/L) leads to overloading of the major high molecular weight carriers and initiates even greater formation of low molecular weight forms of Mn.

## DISCUSSION

Interest in research aimed at studying manganese metabolism in humans and animals, as well as possible adverse effects of manganese exposure, has increased significantly over the last decade. Much attention to this problem is caused by a number of objective reasons, among which the deterioration of the environmental situation takes the main place [16].

The conducted experimental study has revealed that subchronic oral exposure to manganese can lead to disturbance of the balance over chemical elements in the organism of animals.

The decrease in the levels of magnesium, iron and copper in blood serum revealed in the course of the analysis is associated with excessive intake of manganese, a functional antagonist of these metals, into the organism of laboratory animals. The absorption of these elements in the gastrointestinal tract is accomplished by common transport systems and, therefore, the nutritional status of manganese can influence the kinetics of these chemical elements [17, 18].

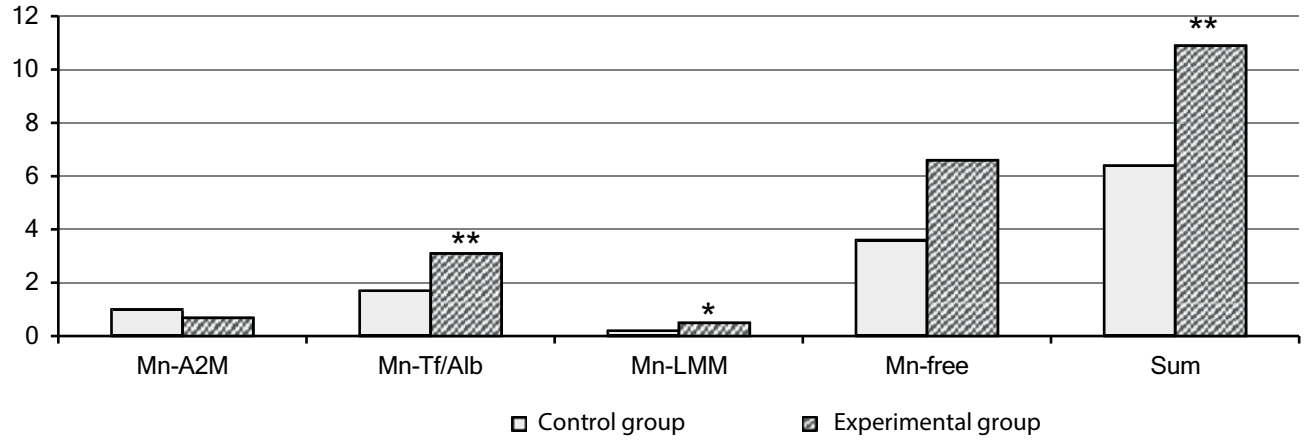


***FIG. 3.***

*Content of manganese fractions in serum of animals (μg/l): data are presented according to median values; * – statistically significant difference between experimental group and control at p ≤ 0.05; ** – statistically significant difference between experimental group and control at p ≤ 0.01*

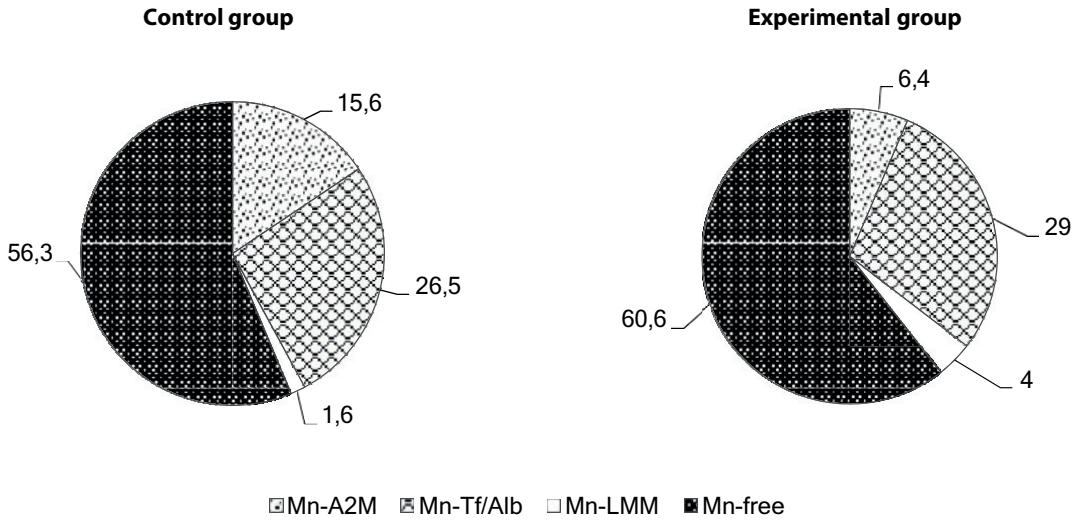


***FIG. 4.***
*Percentage distribution of manganese by fractions (%): * – statistically significant difference between the experimental group and control at p ≤ 0.05; ** – statistically significant difference between the experimental group and control at p ≤ 0.01*

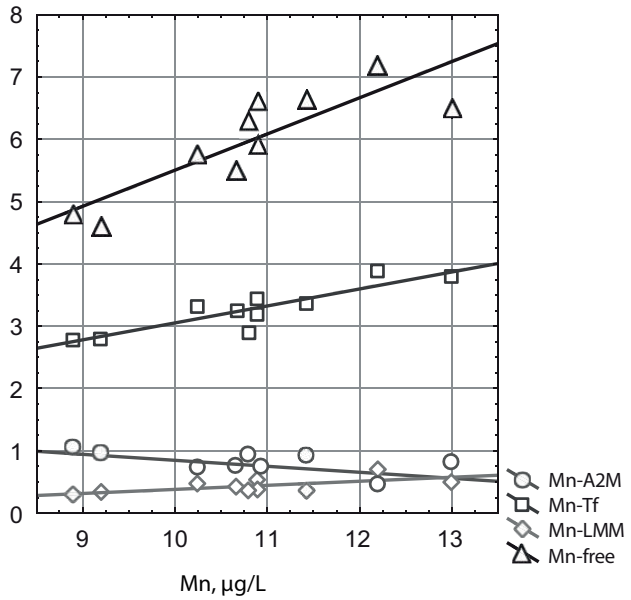


***FIG. 5.***
*Correlation between the total concentration of manganese and its separate fractions in blood serum of animals from the experimental group (n = 10), µg/L*

The observed tendency to increase iodine content may be associated with the blocking effect of manganese ions on iodine uptake by the thyroid gland [19]. Selenium compounds are well known to have an antitoxic effect in poisoning with salts of many heavy metals. The main blood selenoprotein SeP has a high affinity for heavy metals, as a result of which it is able to form complexes with metals and transport them [20, 21]. Specifically, a study by Chinese scientists revealed that mercury exposure affects the concentration of selenium and the distribution of selenoproteins in serum in a dose-dependent manner [22]. This may explain the trend towards higher selenium levels revealed in our study.

The obtained results clearly revealed that chronic exposure to manganese alters iron homeostasis in the systemic circulation, reducing its content in blood serum, as well as in the brain, which is consistent with the studies of foreign scientists [23]. Chemically, this is justified by the fact that manganese and iron are located next to each other in the periodic table, allowing them to compete for binding to transport proteins [24]. Generally, in iron-deficient brain regions, an increased accumulation of manganese occurs, leading to the activation of oxidative stress [25]. Manganese-induced oxidative stress appears likely to cause an increase in the permeability of the blood-brain barrier leading to an increased influx of heavy metals such as lead, mercury and strontium [26].

The development of analytical chemistry techniques has led to the realisation that the total concentration of chemical elements cannot provide complete information as regards their metabolism, bioavailability and possible toxic effects over living organisms. Only by knowledge of the chemical form of an element can provide information about potential chemical and biochemical processes and thus lead to a greater understanding of the toxicity or essentiality of the element [27, 28]. The obtained results of chemical element compound analysis (speciation analysis) clearly demonstrate that prolonged exposure to subchronic doses of manganese can lead to a shift of its metal-ligand forms towards low molecular weight compounds, as well as the inorganic fraction, which may be associated with overloading of other transport molecules. Mn-LMMs are assumed to be the ones capable of crossing the blood-brain barrier and accumulating in the brain, causing neurotoxic effects [29]. A perfusion study conducted by American scientists revealed that a higher

inflow coefficient from blood to the brain is characteristic of Mn-LMM (3-fold higher) compared to Mn-free and Mn-Tf complexes [30].

## CONCLUSION

The obtained results of the study allow us to conclude that subchronic oral exposure to manganese leads to its cumulation in the organism of animals and development of imbalance of a number of macro- and microelements. Such abnormalities can potentiate the development of functional disorders of the body systems. The excessive intake of manganese was revealed to contribute to a statistically significant increase in the content of this heavy metal in blood serum against the background of a statistically significant decrease in the level of calcium, potassium, magnesium, iron and copper. Simultaneously, the level of manganese, lead, mercury and strontium statistically significantly increased in the cerebral cortex; iron and iodine decreased.

The revealed correlations between the content of chemical elements in blood serum and cerebral cortex indicate that changes in serum levels of magnesium and copper may serve as a prognostic sign of disturbance of their homeostasis in the brain.

Redistribution of this metal to its metal-ligand forms occurs against the background of an increase in the gross content of manganese in blood serum. At manganese concentrations in rat serum above 12 μg/L, there is a shift of manganese fraction levels towards low molecular weight LMM compounds, which may be associated with an overload of other transport molecules and may be a new tool for prenosological diagnosis.

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**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# RESTORATION OF ADAPTIVE CARDIOPROTECTION IMPAIRED BY METABOLIC SYNDROME IN RATS BY THE PPARα ACTIVATION

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## ABSTRACT

***Background.*** *It is known that the protective effect of adaptation and conditioning influence is weakened in animals with metabolic syndrome. Metabolic syndrome may be the basis for the failure of cardioprotection in clinical settings.*

***The aim of the study.*** *To identify the relationship between disorder in carbohydrate and lipid metabolism and a decrease in the effectiveness of the infarct-limiting effect of moderate chronic normobaric hypoxia; to check the possibility of correcting reduced cardioprotection by normalizing carbohydrate and lipid metabolism.*

***Materials and methods.*** *The study included 64 Wistar rats. Metabolic syndrome was induced by feeding animals a high-carbohydrate, high-fat diet for 84 days. Chronic normobaric hypoxia was carried out for 21 days in the following mode: 12 % $O_2$ : 0.3 % $CO_2$. Metformin at a dose of 200 mg/kg/day or PPARα agonist WY14643 at a dose of 1 mg/kg/day were added to the drinking water of rats with metabolic syndrome during adaptation period to hypoxia. A 45-minute coronary occlusion and 120-minute reperfusion were performed, and the infarct size was determined. Indicators of lipid and carbohydrate metabolism, leptin, and adiponectin were studied in the blood serum.*

***Results.*** *The infarct-limiting effect of chronic normobaric hypoxia was weakened in animals with metabolic syndrome. Infarct size showed a direct correlation with decreased glucose tolerance and serum triglyceride levels. Using metformin therapy did not lead to the restoration of the infarct-limiting effect of chronic normobaric hypoxia, while the normalization of lipid metabolism with the use of the PPARα agonist WY14643 corrected the impairment of adaptive cardioprotection in rats with metabolic syndrome.*

***Conclusion.*** *The lack of cardioprotection at chronic normobaric hypoxia in rats with metabolic syndrome is associated with impaired carbohydrate and lipid metabolism. The PPARα agonist restores impaired lipid metabolism and adaptive cardioprotection.*

***Key words:*** *myocardium, infarction, adaptation to hypoxia, metabolic syndrome, metformin, PPARα*

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# ВОССТАНОВЛЕНИЕ НАРУШЕННОЙ МЕТАБОЛИЧЕСКИМ СИНДРОМОМ АДАПТАЦИОННОЙ КАРДИОПРОТЕКЦИИ У КРЫС ПУТЁМ АКТИВАЦИИ PPARα

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## РЕЗЮМЕ

***Обоснование.*** *Известно, что протекторное действие адаптационных и кондиционирующих воздействий ослаблено у животных с метаболическим синдромом (МетС). МетС может лежать в основе неэффективности кардиопротекции в клинических условиях.*

***Цель исследования.*** *Выявить взаимосвязь между нарушением углеводного и липидного обмена и снижением эффективности инфаркт-лимитирующего влияния умеренной хронической нормобарической гипоксии (ХНГ); проверить возможность коррекции сниженной кардиопротекции путём нормализации углеводного и липидного обменов.*

***Методы.*** *В исследование включено 64 крысы линии Wistar. МетС вызывали кормлением животных высокоуглеводной высокожировой диетой в течение 84 дней. ХНГ проводили в течение 21 дня в режиме: 12 % $O_2$ : 0,3 % $CO_2$. В питьевую воду крысам с МетС добавляли метформин в дозе 200 мг/кг/ сут. или агонист PPARα WY14643 в дозе 1 мг/кг/сут. в течение адаптации к гипоксии. Проводили 45-минутную коронарооккклюзию и 120-минутную реперфузию, определяли размер инфаркта. В сыворотке крови исследовали показатели липидного и углеводного обменов, лептин, адипонектин.*

***Результаты.*** *Инфаркт-лимитирующий эффект ХНГ оказался ослаблен у животных с МетС. Размер инфаркта показал прямую корреляционную взаимосвязь со снижением толерантности к глюкозе и содержанием триглицеридов в сыворотке крови. Применение терапии метформином не привело к восстановлению инфаркт-лимитирующего эффекта ХНГ, в то время как нормализация липидного обмена при использовании агониста PPARα WY14643 скорректировала нарушение адаптационной кардиопротекции при метаболическом синдроме у крыс.*

***Заключение.*** *Отсутствие кардиопротекции при ХНГ у крыс с МетС связано с нарушением углеводного и липидного обменов. Агонист PPARα восстанавливает нарушенный липидный обмен и адаптационную кардиопротекцию.*

***Ключевые слова:*** *миокард, инфаркт, адаптация к гипоксии, метаболический синдром, метформин, PPARα*

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## INTRODUCTION

A number of strategies have been developed in recent years to protect the myocardium against ischaemic reperfusion injury, such as remote postconditioning, adaptation to chronic moderate hypoxia [1, 2]. However, translating the results of experimental studies into the clinic is difficult since most patients have metabolic disorders [2]. The results of studies conducted in recent years have shown that the metabolic syndrome (MetS), a symptom complex that combines a number of clinical and laboratory indicators of the patient: obesity, arterial hypertension, dyslipidaemia, and carbohydrate metabolism disorders, may underlie the ineffectiveness of adaptation and conditioning in clinical settings [2]. Experimental studies have revealed that a long-term high-fructose diet results in a significant reduction, but not complete prevention, of the beneficial inotropic effects of chronic intermittent hypoxia in an ischaemia-reperfusion model of the isolated heart [3]. The infarct-limiting effect of chronic normobaric hypoxia (CNH) is reduced in animals with diet-induced metabolic syndrome [4]. Meanwhile, it is revealed that the detected decrease in cardioprotection is accompanied by impaired carbohydrate and lipid metabolism [4]. It can be assumed that correction of carbohydrate or lipid metabolism disorders leads to the restoration of CNH cardioprotection that has been lost as a result of metabolic syndrome. Verification of this hypothesis was the aim of the present study.

## METHODS

Experiments were performed on 64 female Wistar rats. The study was approved by the local ethical committee (Protocol No. 201 dated June 30, 2020) and was performed in accordance with the provision of Regulation 2010/63/EU of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes.

The animals were randomly divided into six groups; the initial weights of rats of all groups were equal and were 203 ± 5 g. Group 1 rats ($n$ = 12) were kept on a standard diet for laboratory animals with free access to drinking water.

Group 2 rats were adapted to CNH for 21 days in a chamber with a continuous supply of a gas mixture consisting of 12 % $O_2$, 0.3 % $CO_2$, 87.7% $N_2$, at normal atmospheric pressure [5]. The gas environment was monitored by TCOD-IR and OLC 20 sensors (Oldham France S.A., France) and Bio-Nova-204G4R1 apparatus (Bio-Nova STS (Scientific and Technical Society), Russia) via MX 32 control unit (Oldham France S.A., France). Exposure to hypoxia was stopped 24 h before the start of the experiment.

Group 3 rats ($n$ = 12; diet-induced metabolic syndrome, MetS) were kept for 84 days on a high-carbohydrate, high-fat diet (HCHFD); drinking water was replaced with a 20 % fructose solution. HCHFD composition: proteins – 16 %, fats – 21 %, carbohydrates – 46 % (including 17 % – fructose), cholesterol – 0.125 %, cholic acid – 0.5 % [6]. After HCHFD, animals were kept on a standard diet and normal drinking water for 7 days to exclude exaggerated blood pressure (BP) readings caused by the osmotic effect of fructose consumption.

Group 4 rats ($n$ = 12) were kept for 70 days on HCHFD, after which they were placed in a hypoxic chamber to simulate CNH and continued HCHFD for 2 weeks of adaptation to CNH; the last week of adaptation to CNH, group 4 rats were kept on a standard diet and drinking water without additives.

Group 5 animals ($n$ = 8) as well as group 4 rats were kept on HCHFD, then were exposed to CNH and received the AMPK activator metformin in drinking form at a dose of 200 mg/kg/day throughout the period of adaptation to hypoxia.

Group 6 animals ($n$ = 9) received the PPARα agonist WY14643 at a dose of 1 mg/kg/day in drinking form under the same conditions.

After the end of diet and/or adaptation to hypoxia 1 day before coronary occlusion modelling, all animals had their tail blood pressure measured by non-invasive volumetric plethysmography using MP35 device with NIBP200A pressure measuring device (Biopac System Inc., USA) and glucose tolerance test (GTT) was performed using standard method, area under curve (AUC, area under curve) was calculated. The infarct-limiting effect of adaptation to CNH was evaluated by the infarct size formed during 45-minute coronary occlusion and 120-minute reperfusion in an *in vivo* experiment. Coronary occlusion was performed during the diestrus phase, which was verified by vaginal mucus microscopy. Anesthesia with α-chloralose (80 mg/kg) and artificial lung ventilation with SAR-830/P (CWE, Inc., USA) were used to perform coronary occlusion. Ligation of the left coronary artery was performed 2 mm below the aortic outlet. After 45 min of ischaemia, the ligature was relieved and the onset of reperfusion was verified by hyperaemia of the ischaemic area. The experiment was terminated without taking the animals out of anaesthesia by sampling blood from the external carotid artery. Myocardium was eviscerated, washed through the aorta with physiological solution. The ligature previously applied to the coronary artery area was re-tightened, and the myocardium was stained through the aorta with 5 % potassium permanganate solution to identify the Area at risk, AAR – the area of myocardium exposed to ischaemia. 1 mm thick transverse sections of the left ventricle were made and stained with 1 % 2,3,5-triphenyltetrazolium solution for 30 min at 37 °C, then fixed for 1 day in 10 % neutral formalin solution and scanned (HP Scanjet G2710; HP Inc., USA). The area of myocardial tissue necrosis (infarction size, IS) on sections was revealed as unstained areas with 2,3,5-triphenyltetrazolium. The size of the necrosis area and risk zone was determined planimetrically using the Ellipse 2.02 software (ViDiTo, Czech Republic).

Blood samples were centrifuged at 3000 rpm, serum was collected and stored at -70 °C. Glucose, triacylglycerides, and cholesterol were determined in serum by enzymatic colorimetric method using B-8054, B-8322, and B-8069 kits (Vektor-Best, Novosibirsk, Russia). The content of leptin, adiponectin, corticosterone, and insulin in serum was determined by enzyme immunoassay using SEA084Ra Leptin, SEA605Ra Adiponectin, CEA448Ra Insulin (Cloud-Clone, China); RE52211 Corticosterone (Human, Rat, Mouse) (IBL International GmbH, Germany) kits. Samples were measured using an Infinite 200 PRO microplate reader (Tecan GmbH, Austria).

Statistical data processing was performed using Statistica 13.0 software (StatSoft Inc., USA). The data obtained were verified for agreement of the distribution with the normal law using the Shapiro – Wilk criterion, showed a distribution satisfying the normality criterion, and are presented as mean ± standard error of the mean (M ± SEM). Homogeneity of variance was checked using Levene's criterion. The numerical values of the studied parameters in the groups were comparable in terms of variance, so two-way ANOVA followed by Fisher's posterior criterion was used in their comparison. Correlations between parameters were investigated using Spearman's coefficient. The threshold value of the achieved significance level $p$ was assumed to be 0.05.

## RESULTS

Adaptation of rats to chronic normobaric hypoxia did not affect rat mass and organ mass except for an increase in the mass of the right ventricle of the heart, which is characteristic of the state of chronic hypoxia (Table 1).

**TABLE 1**

**CHANGES IN ORGAN WEIGHTS OF RATS DURING ADAPTATION TO CHRONIC NORMOBARIC HYPOXIA AND METABOLIC SYNDROME**

| Indicators | Control ($n$ = 12) | CNH ($n$ = 11) | MetS ($n$ = 12) | MetS + CNH ($n$ = 12) | MetS + CNH + metformin ($n$ = 8) | MetS + CNH + WY14643 ($n$ = 9) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Weight of rat initial, g | 204.21 ± 1.57 | 201.20 ± 1.98 | 200.00 ± 2.83 | 208.54 ± 1.86 | 212.00 ± 2.97 | 205.82 ± 1.86 |
| Rat weight final, g | 286.37 ± 4.71 | 277.92 ± 6.62 | 298.67 ± 6.55<br>$p_1$ = 0.023 | 279.23 ± 6.98<br>$p_3$ = 0.033 | 284.88 ± 4.89<br>$p_3$ < 0.001 | 273.56 ± 3.25<br>$p_3$ < 0.001 |
| Heart, g | 1.08 ± 0.02 | 1.09 ± 0.04 | 1.27 ± 0.05<br>$p_1$ < 0.001<br>$p_2$ = 0.001 | 1.12 ± 0.02<br>$p_3$ = 0.005 | 1.19 ± 0.02 | 1.20 ± 0.01 |
| Left ventricular mass, g | 0.859 ± 0.016 | 0.797 ± 0.024 | 0.980 ± 0.036<br>$p_1$ < 0.001<br>$p_2$ < 0.001 | 0.818 ± 0.014<br>$p_3$ < 0.001 | 0.850 ± 0.01<br>$p_3$ < 0.001 | 0.840 ± 0.014<br>$p_3$ < 0.001 |
| Right ventricular mass, g | 0.221 ± 0.009 | 0.294 ± 0.023<br>$p_1$ = 0.002 | 0.291 ± 0.021<br>$p_1$ = 0.002 | 0.304 ± 0.013<br>$p_1$ < 0.001 | 0.34 ± 0.02<br>$p_1$ < 0.001 | 0.304 ± 0.013<br>$p_1$ < 0.001 |
| Liver, g | 10.63 ± 0.27 | 10.69 ± 0.32 | 13.12 ± 0.45<br>$p_1$ < 0.001<br>$p_2$ < 0.001 | 11.92 ± 0.41<br>$p_1$ = 0.012<br>$p_2$ = 0.030<br>$p_3$ = 0.027 | 11.67 ± 0.49<br>$p_1$ = 0.01<br>$p_2$ = 0.009<br>$p_3$ = 0.047 | 11.38 ± 0.33<br>$p_1$ = 0.01<br>$p_2$ = 0.005<br>$p_3$ = 0.029 |
| Kidneys, g | 1.83 ± 0.05 | 1.85 ± 0.05 | 2.04 ± 0.05<br>$p_1$ = 0.002<br>$p_2$ = 0.01 | 1.83 ± 0.05<br>$p_3$ = 0.005 | 1.96 ± 0.04 | 1.89 ± 0.07 |
| Abdominal fat, g | 11.32 ± 0.56 | 11.52 ± 0.94 | 15.56 ± 1.24<br>$p_1$ = 0.002<br>$p_2$ = 0.006 | 13.97 ± 1.17<br>$p_1$ = 0.043<br>$p_2$ = 0.088<br>$p_3$ = 0.266 | 14.74 ± 2.86<br>$p_1$ < 0.001 | 14.25 ± 2.15<br>$p_1$ = 0.01 |
| Spleen, g | 0.67 ± 0.05 | 0.54 ± 0.03 | 0.68 ± 0.02 | 0.64 ± 0.03 | 0.69 ± 0.04 | 0.62 ± 0.02 |
| Adrenal glands, mg | 42 ± 3 | 37 ± 2 | 41 ± 1 | 37 ± 1 | 40 ± 1 | 37 ± 1 |

**Note.** $n$ – number of animals in the group; $p$ – statistical significance of differences in relation to the corresponding group (two way ANOVA, Fisher's posterior test).

Keeping rats on a high-carbohydrate, high-fat diet (metabolic syndrome) resulted with an increase in rat weight, abdominal fat mass relative to age-adequate controls, indicating the formation of obesity (Table 1).

In addition, an increase in myocardial mass by 20 %, liver by 30 % and kidneys by 10 % was observed (Table 1). The increase in heart mass in the MetS group was attributable to an increase in both left and right ventricular masses. No statistically significant changes in spleen and adrenal gland masses were observed.

In MetS rats, chronic hypoxia modelling resulted in a decrease in body weight, heart, liver and kidney weights, but not abdominal fat mass to the level of control rats (Table 1). No decrease in the mass of the right ventricular myocardium was observed during the simulation of chronic hypoxia in rats with MetS, indicating the preservation of its hypertrophy, consistent with adaptation to chronic hypoxia [1]. Administration of metformin or WY14643 did not result in statistically significant changes in organ weights in CNH-adapted rats on the MetS background (Table 1).

The formation of metabolic syndrome was characterised by increased blood glucose and insulin content, decreased glucose tolerance (increased area under the curve of glucose dynamics in the glucose tolerance test), insulin resistance (increased HOMA-IR (Homeostasis Model Assessment of Insulin Resistance)), development of hypercholesterolemia, increased triglyceride content in the blood of rats by 1.5 times (Table 2). Adaptation to CNH against MetS background prevented the increase in triglycerides, cholesterol, glucose, glucose and insulin tolerance (HOMA-IR) formation. Along with this, the concentration of insulin in the rat serum under combined modelling of CNH and MetS remained at a high level (Table 2).

Metabolic syndrome caused an increase in serum leptin and adiponectin relative to the control group equally in the groups of non-adapted and CNH-adapted rats (Table 2).

The metabolic syndrome was accompanied by an increase in serum corticosterone levels from 394 ± 6.1 to 475 ± 3.7 ($p_1 < 0.001$), which indicates moderate stress (data are not presented in the table). Adaptation to chronic normobaric hypoxia did not increase corticosterone elevation during MetS. It should be emphasised that the absence of changes in the mass of target organs (adrenal glands, spleen; Table 1) indicates a small severity of the stress response.

**TABLE 2**

**BIOCHEMICAL INDICES OF METABOLIC SYNDROME FORMATION IN RATS UNDER THE INFLUENCE OF METFORMIN AND WY14643**

| Indicators | Control (*n* = 12) | CNH (*n* = 11) | MetS (*n* = 12) | MetS + CNH (*n* = 12) | MetS + CNH + metformin (*n* = 8) | MetS + CNH + WY14643 (*n* = 9) |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Glucose, mmol/L | 4.55 ± 0.34 | 5.02 ± 0.46<br>$p_1 > 0.05$ | 5.32 ± 0.30<br>$p_1 = 0.05$ | 4.36 ± 0.39<br>$p_2 > 0.05$ | 4.6 ± 0.29<br>$p_4 > 0.05$ | 4.95 ± 0.15<br>$p_1 = 0.05$ |
| Glucose tolerance test (AUC) | 709 ± 13 | 723 ± 26<br>$p_1 > 0.05$ | 761 ± 12<br>$p1 = 0.012$ | 725 ± 13<br>$p_2 > 0.05$ | 725 ± 25<br>$p_4 > 0.05$ | 759 ± 22<br>$p_1 = 0.012$ |
| Insulin, pmol/L | 8.02 ± 0.57 | 9.78 ± 0.71<br>$p_1 > 0.05$ | 10.37 ± 0.45<br>$p_1 = 0.05$ | 11.80 ± 0.98<br>$p_1 = 0.002$ | 10.58 ± 1.26<br>$p_4 > 0.05$ | 10.42 ± 0.22<br>$p1 < 0.05$ |
| HOMA-IR | 1.78 ± 0.14 | 2.14 ± 0.14<br>$p_1 > 0.05$ | 2.48 ± 0.13<br>$p_1 = 0.03$ | 2.25 ± 0.26<br>$p_2 > 0.05$ | 2.20 ± 0.30<br>$p_4 > 0.05$ | 2.32 ± 0.11<br>$p_1 = 0.04$ |
| TG, mmol/L | 1.01 ± 0.15 | 1.25 ± 0.16<br>$p_1 > 0.05$ | 1.57 ± 0.29<br>$p_1 = 0.036$ | 1.31 ± 0.15<br>$p_2 > 0.05$ | 1.30 ± 0.16<br>$p_4 > 0.05$ | 1.0 ± 0.09<br>$p_3 = 0.036$ |
| Cholesterol, mmol/L | 4.30 ± 0.44 | 5.34 ± 0.69<br>$p_1 > 0.05$ | 6.71 ± 1.24<br>$p_1 = 0.034$ | 5.62 ± 0.68<br>$p_2 > 0.05$ | 5.56 ± 0.68<br>$p_4 > 0.05$ | 4.51 ± 0.94<br>$p_3 = 0.034$ |
| Leptin, ng/mL | 1.77 ± 0.26 | 1.34 ± 0.11<br>$p_1 > 0.05$ | 5.37 ± 0.74<br>$p_1 < 0.001$ | 5.89 ± 0.55<br>$p_1 < 0.001$<br>$p_2 < 0.001$ | 2.50 ± 0.41<br>$p_3 < 0.001$<br>$p_4 < 0.001$ | 1.25 ± 0.14<br>$p_3 < 0.001$ |
| Adiponectin, μg/mL | 1.77 ± 0.26 | 1.34 ± 0.11<br>$p_1 > 0.05$ | 5.37 ± 0.74<br>$p_1 < 0.001$ | 5.89 ± 0.55<br>$p_1 < 0.001$<br>$p_2 < 0.001$ | 6.96 ± 0.84<br>$p_3 < 0.001$<br>$p_4 = 0.009$ | 8.85 ± 1.17<br>$p_3 < 0.001$ |

**Note.** TG – triglycerides; *n* – number of animals in the group; *p* – statistical significance of differences in relation to the respective group (two-way ANOVA, Fisher's posterior test).

Keeping rats on HCHFD resulted in an increase in systolic (SBP) but not diastolic blood pressure (DBP) (Table 3). Adaptation of rats with metabolic syndrome to hypoxia caused an increase in DBP, while SBP in this group had no statistically significant differences from rats of the control group (Table 3).

Coronary occlusion in all groups of animals induced the formation of a myocardial hypoperfusion zone (risk zone), the size of which was 30–33 % of the left ventricular mass (Fig. 1b; Table 4). 2,3,5-triphenyltetrazolium staining revealed that the infarction size was 46.92 % of the mass of the hypoperfused area. Statistically significant myocardial hypertrophy was observed in the MetS group (Table 1); the mass of myocardial hypoperfusion area (AAR) in this group in absolute terms was statistically significantly greater than in rats without metabolic disorders. However, since the infarct size was considered as the ratio of the necrosis zone and risk zone masses (IS/AAR, %), the infarct size in rats with MetS was not statistically significantly different from that in the control group (Table 4).

**TABLE 3**

**BLOOD PRESSURE IN RATS WITH METABOLIC SYNDROME**

| Indicators | Control ($n$ = 19) | CNH ($n$ = 14) | MetS ($n$ = 15) | MetS + CNH ($n$ = 15) |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| SBP, mmHg | 129.2 ± 2.5 | 129.3 ± 1.9 | 142.0 ± 2.8<br>$p_1$ = 0.014<br>$p_2$ = 0.008 | 137.5 ± 1.6<br>$p_{1.2.3}$ ns |
| DBP, mmHg | 96.1 ± 2.5 | 96.5 ± 2.6 | 98.8 ± 2.2 | 104.7 ± 1.5<br>$p_1$ = 0.039 |

**Note.** $n$ – number of animals in the group; $p$ – statistical significance of differences in relation to the corresponding group (two way ANOVA, Fisher's posterior test); ns – statistically non-significant..

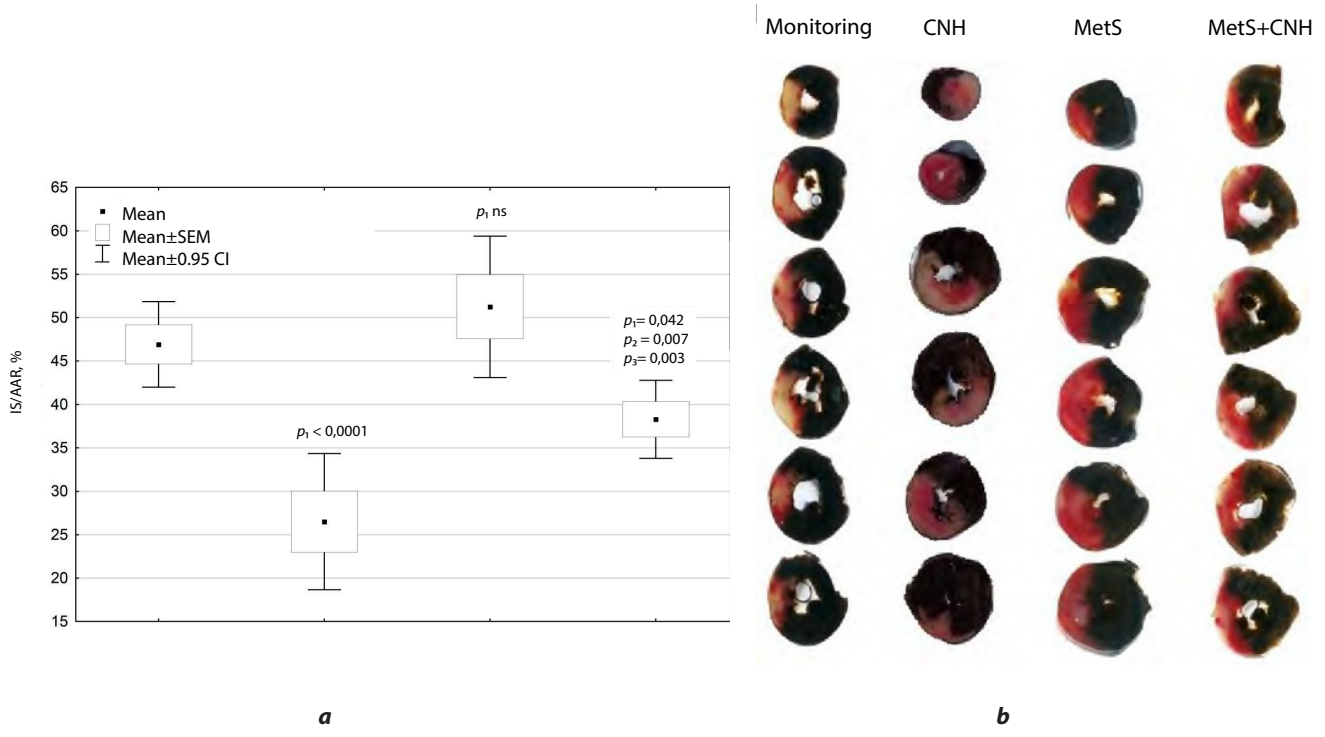


***FIG. 1.***

*CNH infarct-limiting effect in rats with and without metabolic syndrome:* ***a*** *– mean values of infarct size in control group, with MetS, CNH and with the combination of CNH and MetS;* ***b*** *– representative images of myocardial infarction in experimental groups; $p_1$ – statistical significance of differences compared to control group; $p_2$ – statistical significance of differences compared to the group with chronic normobaric hypoxia; $p_3$ – statistical significance of differences compared to the group with metabolic syndrome (two-way ANOVA, Fisher's posterior test); ns – not significant*

Infarct size (IS/AAR, %) in CNH-adapted rats was 43 % smaller than in controls (Fig. 1a, b; Table 4). The obtained data indicate a pronounced CNH infarct-limiting effect. This effect was reduced in animals with MetS: the reduction in infarct size relative to the group of rats with MetS was 25 % (Fig. 1a, b; Table 4). Consequently, we can speak of an attenuation of the CNH infarct-limiting action in rats with diet-induced metabolic syndrome.

Correlation analysis (Spearman's *r*-criterion) revealed weak but statistically significant direct correlations between infarct size and glucose tolerance index (AUC), as well as between infarct size and serum triglyceride content (Table 5).

Metformin therapy (adding it to drinking water at a final dose of 200 mg/kg/day for 21 days of rats adaptation to CNH) did not affect glucose, insulin, triglycerides, cholesterol, glucose and insulin tolerance indices in MetS rats during adaptation to CNH (Table 2). At the same time, under the influence of metformin there was a decrease in leptin content and an increase in adiponectin content relative to the group of rats with metabolic syndrome, including relative to the group of rats adapted to CNH under MetS (Table 2).

Metformin administration did not change left ventricular mass and the size of the risk zone in rats with metabolic syndrome and CNH adaptation; infarct

**TABLE 4**

**EFFECT OF METFORMIN AND WY14643 ON INFARCT SIZE IN RATS AFTER METS AND CNH MODELLING**

| Groups | Left ventricular mass, mg | Risk zone, mg | Necrosis zone, mg | NZ/RZ, % |
|---|---|---|---|---|
| 1. Control ($n$ = 12) | 859 ± 16 | 257.25 ± 20.53 | 120.17 ± 10.34 | 46.92 ± 2.24 |
| 2. CNH ($n$ = 11) | 797 ± 24 | 268.89 ± 18.11 | 73.39 ± 12.29<br>$p_1$ = 0.023 | 26.50 ± 3.52<br>$p_1$ < 0.001 |
| 3. MetS ($n$ = 12) | 980 ± 36<br>$p_1$ = 0.0018 | 329.42 ± 25.51<br>$p_1$ = 0.019 | 170.96 ± 20.04<br>$p_1$ = 0.012 | 51.25 ± 3.70<br>$p_1$ ns |
| 4. MetS + CNH ($n$ = 12) | 818 ± 14 | 241.34 ± 19.07<br>$p_3$ = 0.004 | 93.73 ± 9.92<br>$p_3$ < 0.001 | 38.29 ± 2.04<br>$p_1$ = 0.042<br>$p_2$ = 0.007<br>$p_3$ = 0.003 |
| 5. MetS + CNH + metformin ($n$ = 8) | 850 ± 10 | 250.0 ± 22.38<br>$p_3$ = 0.018 | 103.1 ± 18.99<br>$p_1$ ns<br>$p_2$ ns<br>$p_3$ = 0.0034<br>p4 ns | 39.2 ± 4.75<br>$p_1$ ns<br>$p_2$ = 0.013<br>$p_3$ = 0.016<br>$p_4$ ns |
| 6. MetS + CNH + WY14643 ($n$ = 9) | 865 ± 29 | 247.2 ± 23.5<br>$p_3$ = 0.03 | 72.5 ± 19.2<br>$p_3$ = 0.002 | 29.3 ± 5.7<br>$p_1$ = 0.022<br>$p_2$ ns<br>$p_3$ = 0.003 |

**Note.** $n$ – number of animals in the group; $p$ – statistical significance of differences in relation to the corresponding group (two way ANOVA, Fisher's posterior test); ns – statistically non-significant.

**TABLE 5**

**CORRELATIONS OF BIOCHEMICAL PARAMETERS WITH INFARCT SIZE IN INDUCED METABOLIC SYNDROME AND ADAPTATION TO CHRONIC NORMOBARIC HYPOXIA**

| Indicators | Infarct size NZ/RZ, % | *p* value |
|---|---|---|
| GTT (AUC) | 0.33 | 0.034 |
| Triglycerides, mmol/L | 0.39 | 0.017 |

**Note.** TG – triglycerides; $n$ – number of animals in the group; $p$ – statistical significance of differences in relation to the respective group (two-way ANOVA, Fisher's posterior test).

size was not altered by metformin in rats with combined metabolic syndrome and CNH modelling (Table 4).

Application of PPARα activator WY14643 did not change the indices of carbohydrate metabolism in CNH-adapted rats against MetS background. A decrease in serum triglycerides, cholesterol and leptin was observed under the influence of WY14643 (Table 2). Administration of PPARα activator did not change left ventricular mass and the size of the risk zone in rats with metabolic syndrome and CNH adaptation (Table 4). Simultaneously, the size of the necrosis zone and IS/AAR ratio in rats of this group were lower than in the groups of control animals, in the group of rats with metabolic syndrome and in the group with combined application of metabolic syndrome and CNH (Table 2).

## DISCUSSION OF RESULTS

The results of the study revealed that the use of HCHFD leads to obesity, which is characterised by an increase in body weight and abdominal fat mass, accompanied by hyperglycaemia, impaired glucose tolerance, dyslipidaemia and the development of hypertension. The results obtained allow us to talk about the formation of a metabolic syndrome. Lipid accumulation was previously observed in the myocardium and aorta of rats kept on a diet similar to that used in our study [6]. We found no significant effect of MetS over infarct size, however. There is evidence in the literature of both increased myocardial resistance to ischaemia by a high-carbohydrate diet accompanied by hyperglycaemia [7] and increased infarct size in individuals with metabolic abnormalities such as hyperglycaemia and dyslipidaemia [8]. Accordingly, we can speak about unexpressed myocardial changes in MetS, which do not statistically significantly affect cardiac resistance to ischaemia-reperfusion.

Adaptation to chronic normobaric hypoxia demonstrated a pronounced infarct-limiting effect, which is consistent with the literature and our previous results [1, 5]. Our studies showed that the infarct-limiting effect of CNH is attenuated under MetS conditions. At the same time, adaptation to CNH of MetS rats results in reduction of MetS manifestations such as dyslipidaemia, impaired glucose tolerance. Therefore, we can conclude that CNH significantly but not completely prevents the formation of metabolic disorders, and its protective effect against ischaemic reperfusion injury is reduced. A study by J.J. Zhou et al. (2013) also revealed a decrease in serum glucose content in MetS rats when exposed to chronic hypoxia [3].

The failure of CNH adaptive mechanisms in MetS animals may be a consequence of a number of reasons. Correlation analysis revealed the relationship of infarct size with impaired carbohydrate metabolism. Myocardial insulin resistance in MetS may be considered as mechanisms of this correlation, caused, among others, by the endocrine influence of adipose tissue [9]. Under conditions of adaptation to hypoxia, myocardial metabolism becomes largely dependent from glucose oxidation [10]. Concurrently, decreased activity of the insulin signalling-associated glucose transporter GLUT4 prevents the use of sufficient carbohydrate to sustain the energy status of the cell. Along with this, the hypothesis about the role of myocardial insulin resistance in impairment of CNH infarct-limiting action formation needs additional verification.

The scientific literature discusses the decrease in the activity of adenosine monophosphate-activated protein kinase (AMPK), one of the key enzymes in the regulation of carbohydrate metabolism of the cell, during MetS. It has been revealed that lipotoxicity of fatty acids towards myocardium under high-fat diet is associated with decreased activation (phosphorylation) of AMPK, which leads to the development of myocardial contractility disorders, fibrosis, apoptosis, inflammation and oxidative stress [11].

The fact that AMPK is involved in myocardial defence against hypoxia should be considered [12]. Perhaps inhibition of AMPK signalling by MetS prevents the development of cardioprotection of CNH. These results, however, revealed a lack of metformin efficacy, an AMPK activator, to restore impaired cardioprotection. At the same time, metformin is known to show efficacy in myocardial ischaemia in obese rats induced by a high-fat diet [13]. The mechanisms of cardioprotection in this case are reduction of oxidative stress, anti-apoptotic effect of metformin, reduction of ferroptosis and necroptosis, improvement of contractility, increase of mitochondrial transmembrane potential, decrease of reactive oxygen species formation in mitochondria, and increase of mitochondrial fusion marker OPA1 [13]. According to other authors, however, metformin has no effect upon myocardial ischaemia-reperfusion resistance in rats with streptozotocin-induced diabetes mellitus, including infarct size and post-ischaemic recovery of myocardial contractility [14].

The literature data suggest a definite role in the reduced infarct-limiting efficacy of CNH in rats with MetS for the impairment of the intracellular mechanism of action of adiponectin signalling. It should be noted that our study revealed an increase of adiponectin in rat serum under MetS, which was persisted at a high level when CNH was modelled in these animals. In animals with unaltered carbohydrate metabolism, adiponectin, through interaction with AdipoR1 receptors, stimulates the intracellular APPL1-AMPK response of cardiomyocytes, providing anti-apoptotic and anti-necrotic effects during ischaemia/reoxygenation [15]. This protective effect of adiponectin in myocardial IR has been demonstrated to be reduced in cardiomyocytes of mice with type 2 diabetes mellitus, which may indicate impaired intracellular signalling of this adipokine [15]. Patients with metabolic syndrome were revealed to have decreased expression of both AMPK subunits (α1 and α2) in skeletal muscle, which is correlated with decreased myocyte sensitivity to adiponectin [16].

Additionally, failure of adaptive cardioprotection may be associated with impaired RISK-kinase signalling

during MetS, which is involved in the infarct-limiting effect of CNH. Specifically, it was found that activation of protein kinase B (Akt-kinase) and subsequent cardioprotection in response to preconditioning of rat myocardium did not occur when it was perfused with fatty acids [17]. Stimulation of Akt-kinase phosphorylation restores mitochondrial function impaired when cells are exposed to palmitic acid [9].

It is acknowledged that one of the mechanisms of cardioprotection in chronic hypoxia is the development of the microvascular channel under the influence of hypoxia-induced factors (e.g., HIF1) [18]. This mechanism was revealed to be significantly impaired in patients with MetS, which may be one of the reasons for the impaired formation of cardioprotection [19]. The other mechanism of increased myocardial resistance to ischaemia by HIF-1 factor is the synthesis of microRNA miR-322, which is associated with cytoprotective and antiapoptotic effects of adaptation to hypoxia [20, 21]. This mechanism is affected by MetS [22].

Correlation analysis showed a relationship between infarct size and serum triacylglyceride levels. It can therefore be assumed that disorders of lipid metabolism may be responsible for the failure of adaptive cardioprotection in MetS. PPAR-α receptor is known to be one of the key structures regulating cellular lipid utilisation in cardiomyocytes [23]. PPAR-α is known to be involved in the control of transcription of genes involved in the capture and oxidation of fatty acids in cardiomyocytes [24]. In addition, PGC-1 to PPARα signalling has been also demonstrated to play an important role in the regulation of myocardial resistance to ischemia. For instance, the PPARα agonist clofibrate was revealed to have a direct antiapoptotic effect in ischaemia-reperfusion myocardium of rats with MetS [25], and the PPARα antagonist GW6471 prevented the cardioprotective effect of the cannabinoid anandamide in a model of chronic intermittent myocardial ischaemia (ischaemic cardiomyopathy) in mice [26]. However, the above signalling undergoes significant changes in both diabetes mellitus and chronic hypoxia. A significant decrease in the rate of fatty acid oxidation in rat myocardium was revealed in chronic hypoxia, and, on the contrary, an acceleration of this process in diabetes mellitus induced by the application of a high-fat diet and streptozotocin [10]. The combined state of chronic hypoxia and diabetes mellitus, according to these researchers, reveals a high rate of fatty acid oxidation by mitochondria [10]. No changes in PPARα mRNA in rat myocardium were observed by these authors, neither in isolated exposure to diabetes mellitus and hypoxia, nor in combined pathology. Other publication, however, revealed suppression of the expression of lipid metabolism regulatory protein genes, including PPARα, PPARγ, coactivator 1α (PGC1α), and carnitine palmitoyl transferase 1α (CPT1α), when exposed to hypoxia against a background of diabetes [27]. Meanwhile, PPARα activator WY14643 reduced obesity-induced myocardial lipid accumulation and improved left ventricular systolic function and mitochondrial respiration [27]. These data are consistent with our findings about the infarct-limiting effect of PPARα activator WY14643 in experimental myocardial infarction.

## CONCLUSION

Studies have revealed that diet-induced metabolic syndrome reduces the infarct-limiting efficacy of adaptation to chronic normobaric hypoxia in rats. In this case, the decrease in the effectiveness of chronic normobaric hypoxia is correlated with impaired glucose tolerance and increased triglyceride levels. Correction of carbohydrate metabolism by metformin does not restore the infarct-limiting effect of CNH in metabolic syndrome, whereas the use of PPARα activator normalises lipid metabolism and completely restores the impairment of adaptive cardioprotection in metabolic syndrome in rats.

The obtained data allow us to conclude that for correction of adaptation cardioprotection disorders it is necessary to use means that improve not carbohydrate but lipid metabolism.

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**Conflict of interest**

The authors declare no conflict of interest.

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# PREVENTION AND CORRECTION OF BEHAVIORAL DISORDERS IN RATS WITH METABOLIC SYNDROME USING A COMPLEX PHYTOADAPTOGEN

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## ABSTRACT

***The aim of the study.*** *To assess the possibility of correction and prevention of behavioral disorders in rats with metabolic syndrome using a complex phytoadaptogen (CPA).*

***Material and methods.*** *The experiment was carried out on 30 male Wistar rats randomized into 3 groups: group 1 – control; group 2 – metabolic syndrome (MS); group 3 – treatment of metabolic syndrome using CPA. In groups 2 and 3, animals were on a high-carbohydrate and high-fat diet for 16 weeks. Group 3 received CPA for 14 days in drinking water after 16 weeks of a diet. CPA consists of official tinctures of Glycyrrhiza glabra, Rhodiola rosea, Acantopanax senticosus at a ratio of 1:2:1. Behavior was analyzed through the "open field" test using Realtimer software (Open Science, Russia). Data were analyzed using GraphPad Prism 8.03 software (GraphPad, USA).*

***Results.*** *The experiment proved that metabolic syndrome is accompanied by increased anxiety (decreased horizontal ($p = 0.017$) and vertical ($p = 0.017$) motor activity) and fear (increased periods of immobility ($p = 0.011$)) in the open field. When corrected with a complex phytoadaptogen, the time spent in the open and closed arms of the maze did not differ statistically significantly from the values of similar control indicators.*

***Conclusion.*** *Based on the data obtained in the group 3 (no statistically significant differences with control) – decreased manifestations of fear and anxiety (increased orientation and research activity) – we can talk about the effectiveness of complex phytoadaptogens as an anxiolytic. The mechanisms underlying this result remain to be explored, emphasizing the role of the autonomic nervous system, leptin and ghrelin in behavior and the influence of complex phytoadaptogens on them.*

***Key words:*** *Acanthopanax senticosus, dyslipidemia, Glycyrrhiza glabra, metabolic syndrome, obesity, Rhodiola rosea*

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# ПРОФИЛАКТИКА И КОРРЕКЦИЯ НАРУШЕНИЙ ПОВЕДЕНЧЕСКОЙ АКТИВНОСТИ КРЫС С МЕТАБОЛИЧЕСКИМ СИНДРОМОМ КОМПЛЕКСНЫМ ФИТОАДАПТОГЕНОМ

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## РЕЗЮМЕ

***Цель исследования.*** *Оценить возможность коррекции и профилактики нарушений поведенческой активности крыс с метаболическим синдромом комплексным фитоадаптогеном (КФА).*

***Материал и методы.*** *Эксперимент проводили на 30 крысах-самцах линии Wistar, случайным образом разделённых на 3 группы: группа 1 – контроль; группа 2 – метаболический синдром (МС); группа 3 – лечение метаболического синдрома КФА. В группах 2, 3 животные находились на диете с высоким содержанием углеводов и жиров в течение 16 недель. Группа 3 получала КФА в течение 14 дней с питьевой водой после 16 недель диеты. КФА состоит из официальных настоек Glycyrrhiza glabra, Rhodiola rosea, Acantopanax senticosus в соотношении 1:2:1. Поведение анализировали с помощью теста «Открытое поле» (ОП) с использованием программного обеспечения Realtimer (Open Science, Россия). Данные анализировали с использованием программного обеспечения GraphPad Prism 8.03 (GraphPad, США).*

***Результаты.*** *В ходе эксперимента доказано, что метаболический синдром сопровождается повышенной тревожностью (снижение горизонтальной ($p = 0,017$) и вертикальной ($p = 0,017$) двигательной активности) и страхом (увеличение периодов неподвижности ($p = 0,011$)) в ОП. При коррекции комплексным фитоадаптогеном время пребывания в открытых и закрытых рукавах лабиринта статистически значимо не отличались от значений аналогичных показателей контроля.*

***Заключение.*** *На основании данных, полученных в группе коррекции (отсутствие статистически значимых отличий относительно контроля): снижение проявлений страха и тревожности (повышении ориентировочно исследовательской деятельности), – можно говорить об эффективности комплексных фитоадаптогенов в качестве анксиолитика. Механизмы, лежащие в основе данного результата, ещё предстоит изучить, подчёркивая роль вегетативной нервной системы, лептина и грелина в поведении и влияние на них комплексных фитоадаптогенов.*

***Ключевые слова:*** *Acanthopanax senticosus, дислипидемия, Glycyrrhiza glabra, метаболический синдром, ожирение, Rhodiola rosea*

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## INTRODUCTION

Metabolic syndrome (MS) is becoming a serious medical and social problem worldwide. According to the World Health Organisation, more than 1.9 billion people are overweight [1, 2]. MS is characterised by insulin resistance, hyperglycaemia, dyslipidaemia, hypertension and obesity, and is a pro-inflammatory and pro-thrombotic condition. Visceral fat, a biologically active endocrine and paracrine organ, plays a key role in metabolic syndrome. It produces adipocytokines, the main pro-inflammatory mediators: plasminogen activator inhibitor-1 (PAI-1, plasminogen activator inhibitor-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α). The above cytokines contribute to *low-grade chronic inflammation*, the inflammatory response and reduce insulin receptor sensitivity. Obesity increases the risk of type 2 diabetes mellitus, cardiovascular disease associated with low intensity chronic inflammation and systemic endothelial dysfunction. In recent years, there has been growing interest in exploring the relationship between MS and psychosomatic disorders such as depression and anxiety. The results of studies are conflicting, but pro-inflammatory cytokines have been revealed to cause depressive disorders and depression itself induces eating disorders with subsequent MS formation and has pro-inflammatory properties [3, 4].

In this study, we have examined a new herbal formula that can be effectively used in the treatment and prevention of MS and obesity, a complex phytoadaptogen (CPA), which consists of commonly used phytoadaptogens: golden root (*Rhodiola rosea*), common licorice (*Glycyrrhiza glabra*), spiny eleuterococcus (*Acanthopanax senticosus*). *Glycyrrhiza glabra* and *Rhodiola rosea* have anti-inflammatory and antioxidant effects. *Acanthopanax senticosus* has a pronounced stress-limiting effect. They control stress-activated molecular chaperones (Hsp70), cortisol and nitric oxide (NO). Under stressful conditions, adaptogens modulate the function of the pineal gland [5]. Additionally, the literature suggests that CPAs are able to enhance performance and reduce fatigue, and modulate the inflammatory response in experiment and clinic.

## THE AIM OF THE STUDY

To assess the possibility of correction and prevention of behavioral disorders in rats with metabolic syndrome using a complex phytoadaptogen.

## MATERIALS AND METHODS

The experiment was performed on male Wistar rats (weight 330 ± 20 g; $n$ = 30) obtained from the Rappolovo husbandry (St. Petersburg, Russia). The animals were housed in a room with artificial light (12/12), controlled temperature (21 ± 1 °C) and humidity (50–55 %). Rats were kept in cages (5 animals in each cage), food and water *ad libitum*.

The study was approved by the Ethics Committee of the Institute of Biomedical Research – branch of the Vladikavkaz Scientific Centre of the Russian Academy of Sciences (Minutes No. 7 dated February 20, 2019). The study was conducted in accordance with the ethical standards established by the World Medical Association Declaration of Helsinki (2000).

After the adaptation period (2 weeks), the animals were randomly divided into 3 experimental groups: group 1 – control; group 2 – metabolic syndrome; group 3 – treatment of metabolic syndrome with complex phytoadaptogen. Animals of groups 2 and 3 were on a high-carbohydrate high-fat diet (HCHF). The diet included: 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef fat, 155 g of powdered rat food, 25 g of Hubble, Mendel and Wakeman salt mixture and 50 g of water per kilogram of diet. Additionally, the drinking water for the MS group was supplemented with 25 % fructose [6]. The total feeding time was 16 weeks. The presence of metabolic syndrome in animals was confirmed by biochemical, pathomorphological and functional methods of study according to the applied experimental model [6]. Group 2 rats were removed from the experiment after 16 weeks of feeding to evaluate the progression of pathophysiological changes in the metabolic syndrome. Animals were removed from the experiment by decapitation under thiopental anaesthesia (40 mg/kg).

After 16 weeks of diet, Group 3 rats received a complex phytoadaptogen for 14 days. CPA consists of 40 % alcoholic extracts of *Glycyrrhiza glabra*, *Rhodiola rosea, Acanthopanax senticosus* in the ratio of 1:2:1 respectively. The dose was calculated based on the average daily fluid intake and a factor (×10) for small laboratory animals (0.1 ml/100 g per day).

Behavior was recorded and calculated using computer software for monitoring animal activity (Real Timer, Open Science, Russia) in «open-field» and «elevated plus maze» tests.

The «open-field» test is a square arena with sides equal to 100 cm and a height of 40 cm, divided into equal 25 squares (40 × 40 × 30 $cm^3$). It has been proved that the open field of grey colour fails to reveal intergroup differences in animal behavior, as a specific type of stress behavior emerges, manifested in a mixed anxious-phobic state in animals regardless of their predicted resistance to stress [3]. Considering this, the animals in this experiment were not divided into groups based on their tolerance to stress. Parameters assessed in the open field: vertical activity (number of rears), horizontal activity (distance expressed in squares), number of grooming and defecation acts.

The elevated plus maze is a plus-shaped apparatus with four arms connecting at right angles to each other, as described by Handley and Mitani. The elevated plus maze consists of two closed (30 × 5 × 30 $cm^3$) and two open arms (30 × 5 × 1 $cm^3$) perpendicular to each other

and connected by a central arena (5 × 5 $cm^2$). Closed arms have a high wall (16 cm), whereas open arms have no side wall. Rats were placed in the center platform facing the closed arm. The parameter evaluated is the total time spent in open and closed arms. Entry was recorded when the rat entered the arm with all four limbs.

On the day of testing, the rats were transported to the test room for 2 hours. Each rat was then placed in the same corner of the open-field arena and the elevated plus maze; behavior was recorded for 5 min each in both tests. To avoid the presence of olfactory cues, all equipment (open field, elevated plus maze) was thoroughly cleaned with 20 % ethanol and then wiped with dry paper after each trial. The studies were conducted in the time interval from 9 to 14 hours.

Data analysis was performed using GraphPad Prism 8.03 software (GraphPad, USA). The distribution of continuous variables was tested for normality using the Shapiro – Wilk test. Since in some cases there was no conformity of numerical populations to the principle of normality of distribution and a small number of variants in the compared groups ($n < 30$), nonparametric block statistics was used in the work. The Kraskell – Wallis criterion was used to compare independent data sets. The Wilcoxon criterion was used to compare dependent data sets. Median (25–75‰) values were given as descriptive statistics because of the small number of variants in the sample. A $p$ value < 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

Animal behavior is one way of actively adapting to environmental factors. The animal is stressed when placed in an «open-field» experimental facility, which is reflected in its behavior during the first 4 minutes of the test [3].

Rats with MS (group 2) showed a tendency to decrease horizontal motor activity (HMA) compared to controls (group 1) within 5 min. The number of rears with support (vertical motor activity (VMA)) on the arena wall in group 2 showed statistically significant differences relative to the control ($p = 0.017$) (Table 1). Consequently, in metabolic syndrome, there is a decrease in orienting and exploratory activity (↓HMA and VMA). No statistically significant differences in relation to the control both GDA and VMA were observed during correction with complex phytoadaptogen; the indices were restored within the confidence interval of control, i.e. there was a tendency to normalisation of orientation-research activity.

The main significant manifestation of severe fear in animals with metabolic syndrome in OF was the period of immobility, which was statistically significantly different from control values ($p = 0.011$). This fact confirms the presence of statistically significant differences of this indicator with the control in group 3 ($p = 0.025$). However, the use of CPA resulted in a statistically significant difference from the MS group with respect to periods of immobility ($p = 0.014$).

In the second group, the duration of grooming was statistically significantly different from the control group ($p = 0.011$). When CPA was corrected, statistically significant differences in the number of grooming acts to MS were observed ($p = 0.017$).

Analysis of the parameters of the Elevated Plus Maze test revealed increased anxiety and fear symptoms in the animals. In rats of the second group (MS) relative to controls, there was a decrease in the time spent in the open arm ($p = 0.012$), an increase in the time spent in the dark arm ($p = 0.043$), and the number of head dippings ($p = 0.043$) (Table 1), indicating increased anxiety. When corrected with a complex phytoadaptogen, the time spent in the open and closed arms of the maze did not differ statistically significantly from the values of similar control indicators (Table 1).

## DISCUSSION

Almost all overweight patients have an eating disorder [4]. An understanding of the pathophysiological relationships between stress, neurobiological adaptation and obesity is important for the development of effective prevention and correction methods.

Proceeding from the data obtained during parallel testing of animals during OF and EPM, it is possible to speak about the effectiveness of complex phytoadaptogen as an anxiolytic: normalisation of indices within the confidence interval of control of the test 'open field' (horizontal and vertical motor activity and periods of immobility), test 'elevated plus maze' (time of staying in open and closed arms, number of head dippings, defecation acts) is revealed. This indicates a decrease in the manifestations of fear and anxiety, which was expressed in the activation of orienting and exploratory activity.

The effectiveness of complex phytoadaptogen can be explained by several mechanisms, and first of all it is the effect of increasing adaptation to stress. The autonomic nervous system provides various forms of response to emotional stress – changes in temperature, sweating, cardiovascular and gastrointestinal indices, respiratory rhythmicity. In chronic stress, psychovegetative disorders occur. In normal stress, corticotropin-releasing factor is produced and eventually glucocorticoids are produced [7], which inhibit corticotropin-releasing factor secretion by a feedback mechanism, then the system returns to its initial state. Corticotropin-releasing factor reduces the synthesis of key neuropeptides such as brain-derived neurotrophic factor (BDNF). Chronic stress disrupts the feedback mechanism, glucocorticoids persist for a long time, which leads to neuronal insufficiency in brain structures containing glucocorticoid receptors, for example, the hippocampus, damage to which can lead to a failure of adaptation of the individual under

**TABLE 1**

**CHANGE IN THE ASSESSED INDICATORS IN THE "OPEN FIELD" TEST AMONG ALL EXPERIMENTAL GROUPS**

| Indicators | Control (Group 1) | MS (Group 2) | MS + CPA (Group 3) |
|---|---|---|---|
| | **Open field** | | |
| Number of crossed squares | 58.5 (36; 65.5) | 29.5 (25; 45)<br>$p = 0.017$* | 45 (33.5; 53) |
| Number of rears without support on the open field wall | 9 (5.5; 13) | 2.5 (2; 6)<br>$p = 0.017$* | 7 (3.5; 8.5) |
| Number of rears supported on the open field wall | 7 (3; 8.5) | 6.5 (4; 9) | 5 (3; 7) |
| Immobility period (s) | 20 (15; 32.5) | 60 (45; 75)<br>$p = 0.011$* | 39 (32.5; 45)<br>$p = 0.025$*<br>$p = 0.014$** |
| Grooming acts number | 7 (3.5; 13.5) | 27.5 (20; 40)<br>$p = 0.011$* | 15.5 (14.5; 16)<br>$p = 0.017$** |
| | **Elevated plus maze** | | |
| Time spent in open arms (s) | 50.32 (37.1; 77.7) | 21.83 (10.11; 32.51)<br>$p = 0.012$ | 37 (27.28; 49.35)<br>$p = 0.05$ |
| Time spent in closed arms (s) | 250.8 (228.3; 258.3) | 272.5 (253.4; 287.9)<br>$p = 0.043$ | 228.8 (214.8; 245.1)<br>$p = 0.009$ |
| Head dipping number | 3 (0; 5) | 0 (0; 2)<br>$p = 0.043$* | 0 (0; 0.5) |
| Number of defecations | 3 (2; 3) | 4.5 (2; 6)<br>$p = 0.017$* | 0 (0; 2) |
| Grooming acts number | 2 (2; 3) | 2 (1; 3) | 2 (1; 3) |

**Note.** Results are presented as Me (25‰; 75‰); * – to control; ** – to metabolic syndrome ($p < 0.05$).

subsequent stressors. Accordingly, depressive, anxiety, and somatoform disorders occur [7, 8].

A complex phytoadaptogen can influence animal behavior by several mechanisms. Specifically, CPA increases the production of neurohormones (endorphins, dopamine) in stress by modulating the synthesis of adrenocorticotropic hormone and cortisol in the adrenal glands. In addition, it has a neuroprotective effect [9, 10]. CPA secondary metabolites contribute to the adaptation of cells to stress, which is called the hormesis phenomenon or preconditioning [10].

One other possible mechanism is related to the CPA reduction in the production of inflammatory mediators in the metabolic syndrome. TNF-α is considered as a mediator of insulin resistance and a regulator of energy metabolism in the body. TNF-α has been evidenced to affect insulin receptors and glucose transport, increasing insulin resistance and, consequently, stimulating

leptin secretion [5]. TNF-α increases the production of IL-6, which also inhibits the metabolic effects of insulin by blocking insulin-dependent activation of signalling transducers, insulin-induced glycogen synthesis. Consistent with the cytokine hypothesis, psychosomatic changes with increased cytokines may lead to the induction of indolamine-2,3-dioxygenase to form tryptophan (TRP) catabolites with subsequent decreased availability of TRP and serotonin (5-HT), which also increases anxiety and the development of depressive states [11].

TNF-α has a direct impact on the formation of insulin resistance at the level of hepatocytes. Progression of insulin resistance increases leptin resistance. Leptin («the voice of adipose tissue») regulates eating behavior by acting on the hypothalamic satiety center. Leptin also increases sympathetic nervous system tone; increases thermogenesis in adipocytes; inhibits insulin synthesis; and acts on the cell's insulin receptor to reduce glucose transport. However, in MS, leptin does not fulfil its basic biological functions. The increased anxiety behavior in animals with MS can be explained by the development of leptin resistance in response to a diet rich in carbohydrates and fat, in particular leptin resistance in its main targets, the hypothalamus [12] and ventral tegmental area [13]. This condition possibly develops as a result of decreased leptin receptor gene expression accompanied by reduced signal transduction and/or presumably reduced leptin transport into the cerebrospinal fluid in insulin resistance [14, 15]. It is widely recognised that the amygdala plays a major role in the modulation of anxiety, and dopaminergic receptor mechanisms play an important role in this modulation. To the extent that neurons in the ventral tegmental area are the source of dopamine innervation of the amygdala, it is possible that leptin resistance in it may underlie the anxious behavior observed in our experiment.

Another peripheral hormone that plays an important role in the regulation of homeostatic nutrition is ghrelin. As discussed above in relation to leptin, ghrelin has recently been implicated in stress-induced changes in eating and behavior. Activation of ghrelin signalling pathways in response to chronic stress has been evidenced to be a homeostatic adaptation that helps individuals cope with stress, but at the expense of increased caloric intake. Catecholamines secreted in response to stress appear to directly stimulate ghrelin cells. Like leptin, ghrelin is an efficient modulator of mesolimbic dopaminergic chains [8].

From the third side, chronic low-intensity inflammation leads to increased oxidative and nitrosative damage to neurons, pancreatic and endothelial cells. Inflammatory and nitrosative (O&NS, oxidative and nitrosative stress) pathways are linked in a vicious circle in which immune-inflammatory reactions deplete endogenous antioxidant stores and reactive oxygen intermediates (ROI) activate proinflammatory promoter genes through intracellular signalling cascades such as mitogen-activated protein kinases (MAPKs) and NF-kB; in addition, there is an alteration of insulin-producing cells in the pancreas and an increase in insulin resistance [16].

We can therefore observe a number of overlapping pathophysiological mechanisms contributing to the development of behavioural disorders in chronic low-intensity inflammation against the background of MS, which accounts for the obtained positive effects of complex phytoadaptogen acting at the central (central nervous system), systemic (hormonal regulation) and cellular levels.

The complex phytoadaptogen inhibits transcription factors – NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), receptor activator of nuclear factor kappa-B (RANK, receptor activator of nuclear factor κ B)), and FOXO3, a key transcription factor regulating the cellular response induced by oxidative stress - which results in neurons adapting to stress. Glycyrrhizin, a constituent of *Glycyrrhiza glabra*, reduces oxidative stress by inhibiting the 5'-adenosine monophosphate-activated protein kinase (AMPK) and transcriptional NF-κB pathways and activating AMPK/NRF2 (nuclear factor erythroid 2-related factor 2) signalling [17-19].

*Acanthopanax senticosus* increases the activity of antioxidant enzymes - superoxide dismutase, glutathione peroxidase, catalase - in the liver, thereby reducing the accumulation of ROIs [18]. The most widely known plant-derived antioxidant is *Rhodiola rosea*, which enhances the endogenous antioxidant enzymatic response. Administration of *Rhodiola rosea* extract inhibits the activity of proline dehydrogenase (PDH) and glucose-6-phosphate dehydrogenase (G6PDH, glucose-6-phosphate dehydrogenase). *Rhodiola rosea* extract and its main biologically active substance, tyrosol, increase the activity of superoxide dismutase, which leads to a decrease in the content of free radical oxidation products during adipogenesis [20]. In combination, phytoadaptogens modulate and potentiate the effects of each other, which provides their protective effect.

## CONCLUSION

The results substantiate the idea that a diet high in carbohydrate and fat induces a metabolic syndrome in rats, which contributes to behavioral impairment in the form of impaired orienting and exploratory activity and increased anxiety. Further studies are needed, however, to identify the mechanisms underlying the behavioral changes in leptin resistance that may underlie the anxious behavior observed in our experiment. Biologically active substances of complex phytoadaptogen can be a promising addition to the correction of anxiety, depressive disorders in metabolic syndrome, affecting both through the autonomic nervous system and central structures - targets of leptin and ghrelin, and as protectors of neurons and peripheral tissues from oxidative and nitosative stress, eliminating resistance to insulin, reducing the release of cytokines.

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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## EPIDEMIOLOGY

# EFFECT OF VACCINATION ON MORBIDITY AND MORTALITY FROM COMMUNITY-ACQUIRED PNEUMONIA

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## ABSTRACT

***Background.*** *Community-acquired pneumonia (CAP) remains one of the most common and life-threatening forms of pathology among respiratory diseases.*
***The aim of the study.*** *To identify the features of the epidemiology of community-acquired pneumonia in the conditions of mass immunization against pneumococcal infection in the Irkutsk region.*
***Materials and methods.*** *We conducted a descriptive epidemiological retrospective study. We studied the incidence of community-acquired pneumonia (including community-acquired pneumonia of bacterial and viral etiology) and mortality from this disease in the Irkutsk region for 2011–2022 according to statistical reporting forms No. 2, No. 5, No. 6, C51.*
***Results.*** *The incidence of community-acquired pneumonia during the observation period remained at a high level: the long-term annual average rate among the total population was 627.3 [467.8÷786.8]. At the same time, there was a persistent decrease in the incidence of community-acquired pneumonia of bacterial etiology (Tdecrease = 6.8 %). Incidence rates of community-acquired pneumonia of bacterial etiology were distributed unevenly over the years and the compared population groups. The highest levels were recorded in children in 2018–2019 – 12.3 [10.8÷13.8] and 19.3 [17.8÷20.8], respectively. A decrease in the mortality rate from community-acquired pneumonia among children, adults and in the general population in 2020 was shown, with a subsequent increase in the rate among adults and the general population by 2.5 times. Against the background of ongoing immunization of the population against pneumococcal infection, there is a statistically significant decrease in the incidence of community-acquired pneumonia, including community-acquired bacterial pneumonia, and mortality from community-acquired pneumonia among different population groups.*
***Conclusion.*** *Despite the high incidence of community-acquired pneumonia, a statistically significant decrease in the incidence of community-acquired pneumonia of bacterial etiology has been shown among children and adults. The decrease in mortality from community-acquired pneumonia has continued since the introduction of immunization against pneumococcal infection. The results of the study can be used to optimize epidemiological surveillance and epidemiological control of community-acquired pneumonia at the regional level.*

***Key words:*** *community-acquired pneumonia, bacterial pneumonia, pneumococcal pneumonia, viral pneumonia, morbidity, mortality, vaccination*

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# ВЛИЯНИЕ ВАКЦИНАЦИИ НА ЗАБОЛЕВАЕМОСТЬ И СМЕРТНОСТЬ ОТ ВНЕБОЛЬНИЧНЫХ ПНЕВМОНИЙ

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## РЕЗЮМЕ

***Обоснование.*** *Внебольничные пневмонии (ВП) остаются самой распространённой и угрожающей жизни формой патологии среди болезней органов дыхания.*

***Цель исследования.*** *Выявить особенности эпидемиологии внебольничных пневмоний в условиях массовой вакцинации против пневмококковой инфекции в Иркутской области.*

***Материалы и методы.*** *Проведено описательное эпидемиологическое ретроспективное исследование. Изучены заболеваемость ВП (в том числе бактериальной и вирусной этиологии) и смертность от них в Иркутской области за 2011–2022 гг. по данным статистических отчётных форм № 2, № 5, № 6, С51.*

***Результаты.*** *Заболеваемость ВП за период наблюдения сохранялась на высоком уровне: среднемноголетний показатель среди совокупного населения составил 627,3 [467,8÷786,8]. При этом отмечалось стойкое снижение уровня заболеваемости ВП бактериальной этиологии (Тсниж. = 6,8 %). Показатели заболеваемости внебольничными пневмококковыми пневмониями были распределены неравномерно по годам и сравниваемым группам населения. Наибольшие уровни регистрировались у детей в 2018–2019 гг. – 12,3 [10,8÷13,8] и 19,3 [17,8÷20,8] соответственно. Показано снижение уровня смертности от ВП среди детей, взрослых и совокупного населения в 2020 г. с последующим ростом показателя среди взрослых и совокупного населения в 2,5 раза. На фоне проводимой вакцинации населения против пневмококковой инфекции (ПИ) отмечается статистически значимое снижение уровня заболеваемости ВП, в том числе внебольничными бактериальными пневмониями и смертности от ВП среди разных групп населения.*

***Заключение.*** *Несмотря на высокие уровни заболеваемости ВП, показано статистически значимое снижение заболеваемости ВП бактериальной этиологии среди детей и взрослых. Снижение смертности от ВП продолжилось с начала введения вакцинации против пневмококковой инфекции. Полученные результаты исследования могут быть применены для оптимизации эпидемиологического надзора и эпидемиологического контроля за ВП на региональном уровне.*

***Ключевые слова:*** *внебольничные пневмонии, бактериальные пневмонии, пневмококковые пневмонии, вирусные пневмонии, заболеваемость, смертность, вакцинация*

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## INTRODUCTION

Community-acquired pneumonia (CAP) is one of the most common infectious diseases [1, 2]. High levels of morbidity and mortality, polyetiology, and difficulties in laboratory diagnosis necessitate optimisation of epidemiological surveillance and approaches to the prevention of this pathology [3, 4]. *Streptococcus rpeimopiae* (*S. rpeimopiae*) is the dominant agent in respiratory tract lesions [5]. The World Health Organisation estimates that pneumococcal disease (PD) is responsible for more than 1.5 million deaths each year, and almost half of these are in children. An unfavourable factor hindering the effectiveness of treatment is the progressive antibiotic resistance of strains of this pathogen [3, 6]. The diversity of clinical forms, high prevalence of PD determines the relevance of the problem of their prevention [5]. With the epidemic spread of viral infections (influenza, COVID-19), the relevance of CAP, including bacterial etiology, remains [7]. Viral infections have been confirmed to be the trigger mechanism for colonisation of the respiratory tract by pathogenic bacterial flora [8].

Vaccine prophylaxis is recognised as an effective strategy for the prevention of CAP [5]. Vaccination against influenza, Haemophilus influenzae type B, measles, pertussis, PD, COVID-19, implemented within the framework of Immunization Schedule (IS) of different countries, is aimed at reducing CAP morbidity and mortality among different population groups. Scheduled vaccine prophylaxis of pneumococcal infection on the territory of the Russian Federation was included in the IS one of the last, in 2014. The accumulated experience of the scheduled vaccination demonstrates the epidemiological and prophylactic efficacy of this measure in some regions [9-11].

In the Irkutsk region, the incidence rate of CAP in the period 2011–2019 exceeded that of the Russian Federation (RF) by 1.5 times [12]. Specific PD prophylaxis is carried out within the framework of Order No. 1122н (Annexes 1, 2). It is extremely relevant to study changes in the level of morbidity and mortality caused by CAP at the level of the constituent entity of the Russian Federation in the conditions of scheduled vaccine prophylaxis against pneumococcal infection.

## THE AIM OF THE STUDY

To identify the features of the epidemiology of community-acquired pneumonia in the conditions of mass immunization against pneumococcal infection in the Irkutsk region.

## MATERIALS AND METHODS

We conducted a descriptive epidemiological retrospective study. We studied the incidence of CAP (viral and bacterial, including pneumococcal, etiology) and mortality from their occurrence in the Irkutsk region for 2011–2022 by continuous samples of reporting forms № 2 "Information about infectious and parasitic diseases", C51 "Distribution of the dead by sex, age and causes of death". The volumes of vaccination against pneumococcal infection among the population are presented according to the data of forms No. 5 "Information on preventive vaccinations" for 2013–2022; No. 6 "Information on contingents vaccinated against infectious diseases" for 2016–2022.

CAP incidence and mortality of different population groups (child, adult, aggregate) are presented by periods: 2011–2019 – period before the spread of COVID-19; 2020 – COVID-19 pandemic onset year; 2021–2022 – years of COVID-19 spread.

To assess the extent to which immunisation against PD influences the incidence of CAP, community-acquired bacterial pneumonia (CABP) and community-acquired pneumococcal pneumonia (CAPP) and CAP mortality, age stratified analyses were performed in children (under 17 years), adults (18 years and older) and the general population.

Descriptive epidemiological methods were used to study long-term dynamics of CAP morbidity, including bacterial etiology, CAPP and CAP mortality, distribution of morbidity by age groups of the population. Calculation of relative indices, average annual growth/decline rates ($T_{growth}/T_{decrease}$), regression equations, Spearman correlation coefficients, data analysis and their graphical representation were performed using MS Office Excel 2010 (Microsoft Corp., USA).

Statistical significance of differences in relative values was calculated using confidence intervals with a significance level of $p \leq 0.05$ (95% CI).

## RESULTS

The CAP incidence among different age groups of the population in the Irkutsk region remained at a high level. For the period 2011–2019, the incidence rate for children (0-17 years) exceeded that of adults and the general population by a factor of 2.3 and 1.7, respectively. In 2020, a significant increase in the CAP incidence rate among adults was registered. In the subsequent period (2021–2022), the incidence rate among adults decreased but exceeded that among children (Table 1).

In analysing the CAP structure among laboratory-confirmed cases within follow-up, the predominance of CAP cases of bacterial etiology was observed – 78.4 %; the exceptions were 2020 and 2022, where the share of pneumonias of viral etiology was 84.2 % and 73.5 %, respectively.

The dynamics of CAP morbidity incidence of bacterial etiology during the observation period was characterised by a decreasing trend among children and adults. The incidence rate in children was

higher and declined at a higher rate than in adults; the average annual rates of decline were 9.3 per cent and 5.8 per cent, respectively. In the multi-year follow-up of child morbidity, the period 2018–2019 was characterised by a significant increase in indicators: the average level was 179.7 [125.6÷233.8] per 100,000 population, in 2018 the incidence increased by 14.0 % compared to the previous period, in 2019 – by 35.0 %. In 2021, there was a significant decrease in the incidence rate, the indicator was 21.4 [12.4÷30.4] per 100 thousand population (the lowest level for the observation period); in 2022, a statistically insignificant increase in the incidence rate was registered – 30.1 [25.4÷34.8] per 100 thousand population. Similar trends were observed in the follow-up of adult morbidity. CAP incidence of viral aetiology differed significantly between the compared groups. Specifically, among the adult population, the period 2011–2018 was characterised by a consistently low incidence rate, with a long-term average annual indicator (LTAAI) of 9.4 [0÷18.4] per 100,000 population. An increase in the rate to 18.8 [16.9÷20.7] per 100 thousand population was observed from 2019, which continued in 2020 – to 721.9 [709.7÷734.1] per 100 thousand population. In 2021, the incidence rate was observed at the 2019 level with a further increase in 2022. In the long-term follow-up of the CAP incidence of viral aetiology in children, the levels and periods of incidence were distributed similarly to those of bacterial CAP: periods of decrease and growth alternated with each other. In 2020, the CAP incidence of bacterial and viral etiology was recorded at 80.2 [72.6÷87.8] and 86.5 [78.3÷94.7] per 100,000 population, respectively. The lowest incidence rate for the observation period was recorded in 2021 – 3.2 [1.6÷4.8] per 100 thousand population; in 2022 there was another rise in the incidence rate (statistically significant), the rate was 54.0 [47.6÷61.6] per 100 thousand population (Fig. 1).

The share of CAPP in the structure of bacterial CAP was insignificant. In particular, the minimum level was observed in 2013 and amounted to 0.4 %, the maximum in 2021 (11.0 %). According to the long-term average annual values for the analysed period, the CAPP share was 3.1 %, with a lower average for the period 2013–2019 than for the period 2020–2022: 2.5 % and 4.6 % respectively.

Since 2013, laboratory-confirmed cases of pneumococcal pneumonia have been registered in the Irkutsk region in reporting form No. 2. For the period 2013–2022 LTAAI in the general population was 3.2 [1.9÷4.5] per 100,000 population, in children and adults 5.5 [2.0÷9.0] and 2.6 [1.4÷3.8] per 100,000, respectively (differences were not statistically significant). Analysis of the comparison of CAPP long-term incidence rates in adults and children showed an uneven distribution of indicators over the years with multidirectional trends. The following periods can be distinguished in the follow-up of the CAPP incidence rate among children: 2013–2017 – decrease in the incidence rate (the average annual rate of decrease was 5.8 %, regression coefficient -0.6); 2018–2019 – significant growth of indicators (growth rate ($T_{growth}$) = 21.5 %; incidence rate – 12.3 [10.8÷13.8] and 19.3 [17.8÷20.8], respectively); 2020–2022 – sharp decrease in the incidence rate (decrease rate – 27.8 %). Over the period 2013–2022, the CAPP adult incidence trends show an excess of 1.3 times the LTAAI in 2015 and 2018 and 3.7 times the LTAAI in 2021. In 2022, the incidence of pneumococcal pneumonia in the comparison groups was 0.5 per 100,000 (Fig. 2).

Among children of different age groups, the highest incidence rates of CAPP were registered in the age groups under 1 year and 1–2 years, LTAAI was 12.1 [0÷31.8] and 17.1 [4.1÷30.1] per 100,000 population, respectively. It is worth noting that in 2018–2019, an increase in incidence rates was observed among children of all age groups. The maximum incidence rate was observed in 2019 in children under 1 year old and was 103.7 per 100,000 population (Table 2).

One of the important areas that characterise the epidemic process manifestations of an infectious disease

**TABLE 1**

**COMMUNITY-ACQUIRED PNEUMONIA INCIDENCE AMONG DIFFERENT POPULATION GROUPS COMPARED BY PERIODS (PER 100,000 POPULATION)**

| Population groups | 2011-2019 (LTAAI) | 2020 | 2021-2022 (LTAAI) |
|---|---|---|---|
| Total population | 528.4* [437.3÷619.5] | 1400.5* [1385.8÷1415.2] | 685.7* [454.8÷916.6] |
| Children under 17 years old | 928.5* [786.8÷1070.2] | 732.4 [710.6÷754.2] | 569.6* [488.3÷704.7] |
| Adult population (persons over 18 years of age) | 411.3* [325.5÷497.1] | 1613.1* [1595.1÷1631.1] | 711.9* [350.7÷1073.1] |

**Note.** LTAAI – long-term average annual indicator; * – statistically significant differences by observation periods.

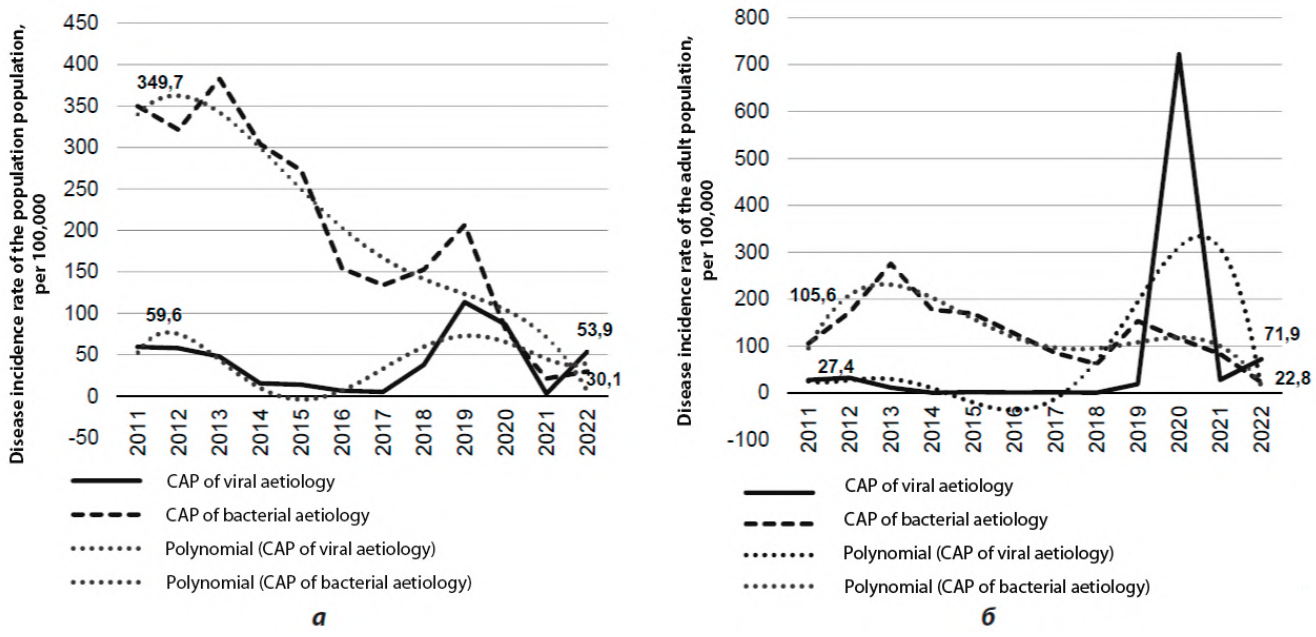


***FIG. 1.***
*Long-term follow-up of the incidence of community-acquired bacterial and viral pneumonia in children (a) and adults (б) in the Irkutsk region for the period 2011-2022 (per 100,000 population)*

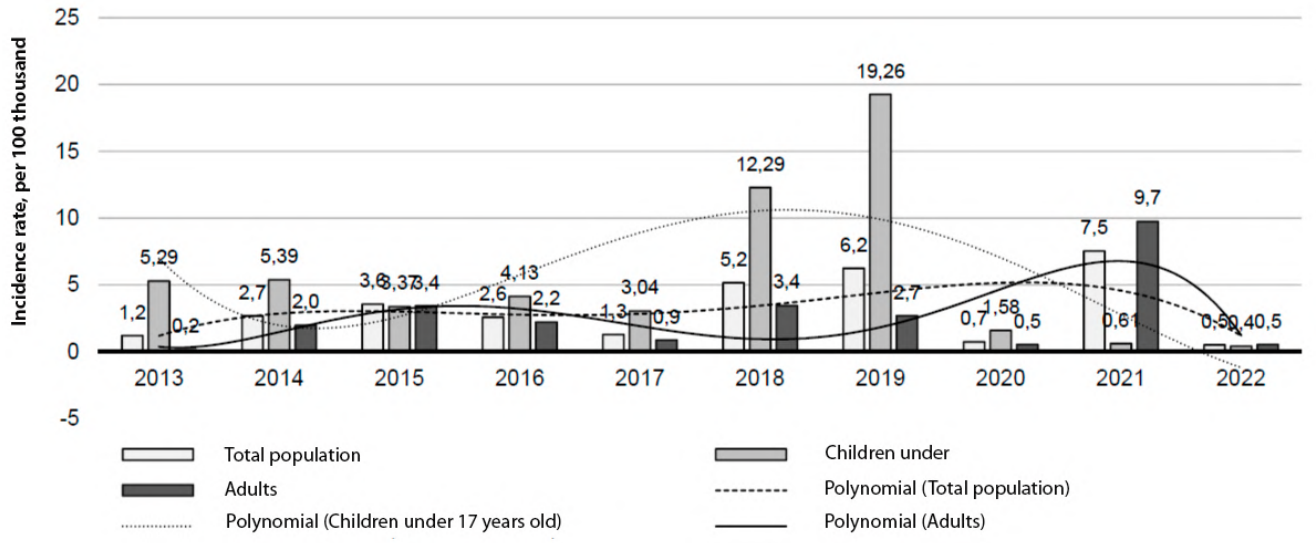


***FIG. 2.***
*Long-term incidence rate of community-acquired pneumococcal pneumonia in children, adults and the total population of the Irkutsk region for the period 2013-2022 (per 100,000 population)*

is the assessment of the mortality rate of the population. In 2020, CAP mortality rates were lower than 2011–2019 levels in the general population, children, and adults by a factor of 1.7, 7.8, and 1.7, respectively. In contrast, in 2021 among adults and the total population, the rate was 2.5 and 2.4 times higher than the 2020 level, respectively; and the 2011–2019 level was 1.4 times higher than the 2020 level. The specific volume of bacterial CAP in the structure of CAP mortality during the observation period varied from 7.4 to 18.1 %, with the minimum level being observed in 2020–2021. CABP mortality rates among the compared groups ranged from 0.5–4.8 per 100,000 population. Among children for different periods

the rate did not exceed 1.0 per 100 thousand population, among adults – a decrease in the mortality rate in 2020–2021 was observed (Table 3).

The coverage of the Irkutsk region total population with preventive vaccinations during the analysed period was at the level of 4.0–8.0 %. Vaccination rates among children increased annually from 14.0 % to 22.0 %. The total number of people vaccinated against PD in the total population for 2013–2022 was 401,812 (17.0 % of the 2022 population), among them 262,733 children (53.4 % of the 2022 child population).

The number of people vaccinated against pneumococcal infection was unevenly distributed by year. The CAP incidence rate among children from the onset of vaccination till 2022 decreased 1.7-fold from 1266.6 [1254.9÷1278.3] to 715.3 [692.0÷738.6] ($p < 0.05$; Fig. 3). During the period analysed, there was an inverse correlation between the number of children vaccinated and the number of children sickened with CAP, CABP and CAPP ($\rho = -0.555$; $\rho = -0.139$; $\rho = -0.382$, respectively); the differences were not statistically significant ($p > 0.05$). Unlike children, among the adult population, the incidence of VP increased 1.2 times over the same period – from 436.7 [427.5-445.9] to 527.6 [517.3-537.9] ($p < 0.05$). A statistically non-significant direct correlation was revealed between the number of vaccinated persons and the number of CAP, CABP and CAPP cases ($\rho = 0.636$; $\rho = 0.345$; $\rho = 0.227$, respectively; $p > 0.05$).

**TABLE 2**

**FOLLOW-UP INCIDENCE RATE OF COMMUNITY-ACQUIRED PNEUMOCOCCAL PNEUMONIA IN CHILDREN OF DIFFERENT AGE GROUPS IN 2013–2022 (PER 100,000 ELIGIBLE CHILDREN; 95% CI)**

| Age groups | Periods | | | LTAAI |
|---|---|---|---|---|
| | 2013-2017 | 2018-2019 | 2020-2022 | |
| Up to 1 year | 2.7 [1.1÷4.3] | 51.8 [0÷153.3] | 1.8 [0÷4.1] | 12.1 [0÷31.8] |
| 1-2 years | 8.7* [5.0÷12.4] | 57.2* [54.5÷59.9] | 0.9 [0÷7.7] | 17.1 [3.8÷30.4] |
| 3-6 years old | 3.2 [2.5÷4.9] | 8.2 [1.6÷14.8] | 0 | 3.2 [1.2÷4.3] |
| 7-14 years old | 3.7 [0.8÷6.6] | 5.1 [0÷11.7] | 0.5 [0.2÷0.8] | 3.0 [0.9÷5.1] |
| 15-17 years old | 3.2 [0.5÷5.9] | 2.9 [0÷8.5] | 0.6 [0÷1.3] | 2.3 [0.8÷3.8] |

**Note.** * – statistically significant differences by observation periods.

**TABLE 3**

**MORTALITY CAUSED BY COMMUNITY-ACQUIRED PNEUMONIA, INCLUDING BACTERIAL PNEUMONIA, AMONG DIFFERENT POPULATION GROUPS COMPARED BY PERIODS (PER 100,000 POPULATION)**

| Population groups | 2011-2019 (LTAAI) | | 2020 | | 2021 | |
|---|---|---|---|---|---|---|
| | CAP | CABP | CAP | CABP | CAP | CABP |
| Total population | 37.8* [35.4÷40.2] | 3.8* [3.0÷4.6] | 21.4* [19.6÷23.2] | 1.7* [1.1÷2.3] | 51.8* [48.9÷54.7] | 2.2 [1.6÷2.8] |
| Children under 17 years old | 3.9* [2.3÷5.5] | 0.4 [0÷0.8] | 0.5* [0÷1.1] | 0 | 1.2 [0.4÷2.0] | 0.5 [0÷1.1] |
| Adult population (persons over 18 years of age) | 47.8* [44.7÷50.9] | 4.8* [3.8÷5.8] | 27.2* [24.8÷29.6] | 2.2* [1.6÷2.8] | 67.7* [64.0÷71.4] | 3.2 [2.2÷4.2] |

**Note.** * – statistically significant differences by observation periods.

One of the objectives of vaccine prophylaxis at the present stage is to reduce mortality caused by infections. Over the period 2011–2019 in the total population, CAP mortality decreased from 56.1 [53.2÷59.0] to 15.2 [13.6÷16.8] per 100 thousand (differences are statistically significant). An inverse correlation of medium strength between the number of vaccinations and mortality rate was observed for each year (ρ = -0.614; $p > 0.05$). In 2020–2021, an increase in the indicator is observed with the maximum value in 2021 – 51.8 [48.9÷54.7] per 100 thousand population. Among children, there was a pronounced downward trend in this indicator: the average annual rate of decline was 8.4 %, regression coefficient -0.82. In 2020, the indicator was 7.6 times lower than in 2011. There was a weak inverse correlation between the number of vaccinated and CAP deaths (ρ = -0.214; $p > 0.05$) (Fig. 4).

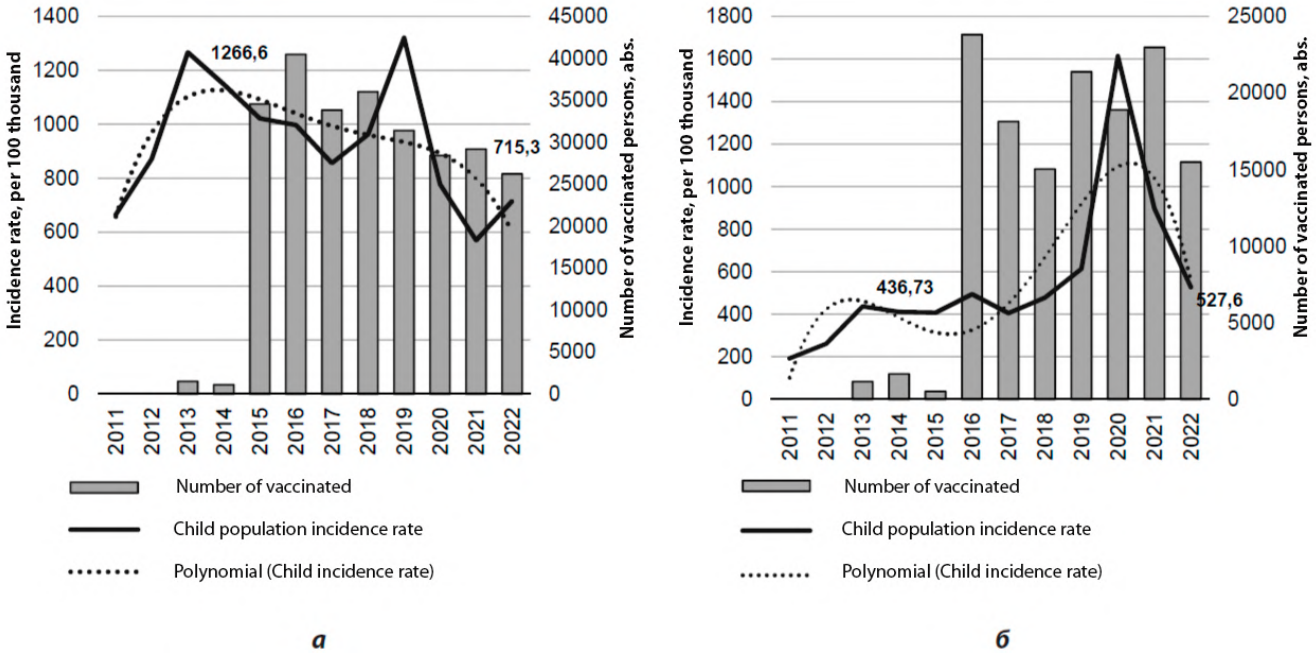


***FIG. 3.***
*Long-term incidence followed-up of community-acquired pneumonia and the number of children (a) and adults (б) vaccinated against pneumococcal infection in the Irkutsk region for the period 2013-2022 (per 100,000 population)*

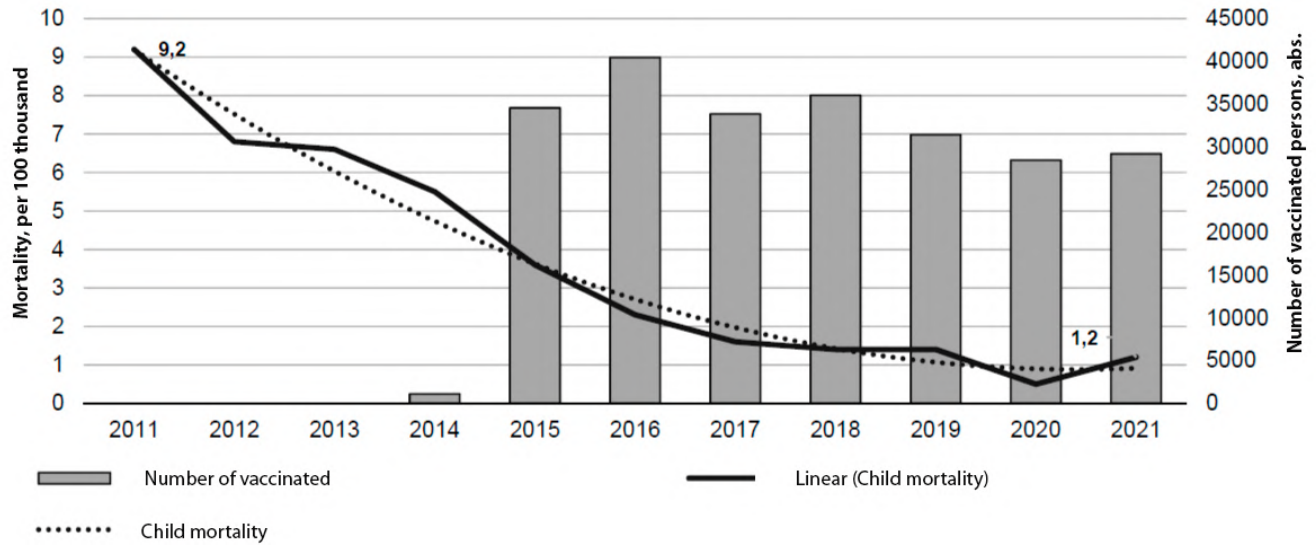


***FIG. 4.***
*Long-term dynamics of mortality caused by community-acquired pneumonia and the number of children vaccinated against pneumococcal infection in the Irkutsk region for the period 2011-2021 (per 100,000 population)*

## DISCUSSION

The incidence of community-acquired pneumonia in Irkutsk region was observed at a high level in different periods of observation. Against the background of the COVID-19 pandemic, the incidence rate has increased and the age groups at risk have changed, which has also been observed in studies [3, 7, 12]. In the Irkutsk region, as well as in the Russian Federation as a whole, in 2020 the maximum CAP incidence rate among adults was observed, which is associated not only with the selective effect of the SARS-CoV-2 virus, but also with the approaches to statistical recording and registration of COVID-19 infection: this disease was not registered as an independent nosological form, and all manifestations of the new coronavirus infection were counted either as community-acquired pneumonia or in the group of acute respiratory infections of the upper respiratory tract [12]. The CABP incidence was higher compared to viral among different populations. Accordingly, the long-term average annual indicator for CAP of bacterial etiology exceeded that for CAP of viral etiology among children and adults by 4.8 and 1.7 times, respectively; the periods of growth and decline in the multi-year follow-up coincided. This distribution of CAP by etiological agent is explained by a wide range of typical and «atypical» bacteria and their associations (*pneumococci, Klebsiella, chlamydia, mycoplasmas, Staphylococcus aureus, Pseudomonas bacillus*, etc.) [1, 3].

The proportion of laboratory-confirmed pneumococcal pneumonia and pneumococcal infection, according to different authors, varies between 4–45 % [3, 6, 13]. The results of this study reveal a very low proportion of laboratory-confirmed CAPP cases (0.4–6.4 %) among the reported cases, with a significant increase (11.0 %) during the period of COVID-19 infection, which may be associated with an increase in the number of laboratory tests performed [12].

An increase in the incidence rate of CAPP in children was revealed in 2018–2019; the average annual rate of increase was 21.5 %. In the region, this period was characterised by an increase in the incidence of influenza and respiratory tract infections among the population [12]. The increased incidence of CAPP in adults in 2021 is also likely to be associated with the adherence of bacterial infections in the spread of COVID-19 [7].

Against the background of increased incidence rate, a high level of mortality from CAP persisted. Among adults, the rates were significantly higher at different periods and ranged from 27.2 to 67.7. No data is available to study the dynamics of mortality from pneumococcal pneumonia according to statistical reporting forms due to the lack of information in them. According to the results of sample studies conducted earlier in the Irkutsk region [14], the proportion of laboratory-confirmed cases of fatal pneumococcal pneumonia was 30 %.

According to the Order of the Ministry of Health of the Russian Federation No. 1122н, children aged 2 and 4.5 months should be vaccinated twice against PD, followed by revaccination at 15 months of age. For epidemic reasons, vaccination is indicated for children 2–5 years of age and adults at risk (persons over 60 years of age suffering from chronic lung diseases, persons over working age living in social service organisations and persons subject to military conscription). Pneumococcal conjugate vaccines (PCV) have been recommended for routine immunisation of children, and their use helps to reduce the incidence and carriage of PD in the general population. A single administration of polysaccharide pneumococcal vaccine (PPV) or sequential administration of PCV and PPV at 1-year intervals is recommended for immunisation of adults at risk, depending on the presence or absence of immunocompromising conditions and comorbidities [3, 5, 15, 16].

In the region, prophylactic vaccination coverage against PD has increased among the general population and children to 8.0 % and 22.0 %, respectively, since the introduction of vaccination. The effect of vaccination on the incidence of CAP and CAPP has been shown in studies [5, 9, 15]. The results of the study revealed a statistically significant reduction in bacterial CAP in different population groups (105.7 [101.2÷110.2] and 30.1 [27.6÷32.6] among adults; 349.8 [332.4÷367.2] and 22.8 [18.7÷26.9] among children). CAPP incidence rates were recorded an order of magnitude lower and were unevenly distributed in follow-up. There was an inverse correlation with the number inoculated ($\rho = -0.382$; $p > 0.05$). One of the reasons for the increased incidence of adult CAP against the background of vaccination against PD and other infectious diseases (influenza, COVID-19) may be the difficulty in organising vaccination work among these populations, including an increase in preventive vaccination coverage rates as a consequence of low vaccination adherence [17].

The decrease in mortality from pneumonia against the background of vaccination is clearly traceable and consistent with the data of other researchers [1, 3, 16]. An inverse significant correlation was observed between the number of vaccinated persons and the number of CAP deaths in the general population and among children by year (differences were not statistically significant; $p > 0.05$), which is probably associated with the short period of observation. No doubt, other therapeutic, diagnostic and preventive measures also have an impact on the reduction of CAP mortality. However, vaccination of the population against PD should not be ruled out.

## CONCLUSION

Despite high levels of CAP morbidity, a statistically significant decrease in the incidence for CAP

of bacterial aetiology among different age groups of the population was revealed. The decline in CAP mortality has continued since the introduction of PD vaccination. However, the insignificant share of pneumococcal pneumonias in the structure of CAP, uneven distribution of morbidity rates by population groups, and lack of registration of CAPP as a cause of death in statistical forms of Rosstat (Russian Federal State Statistics Service) make it difficult to assess the objective epidemiological situation and the effectiveness of vaccination.

The results of the study can be used at the regional level to optimize epidemiological surveillance, primarily in terms of microbiological monitoring of cases of illness and death from CAP, CABP, CAPP; as well as epidemiological control of PD, aimed at increasing the coverage of preventive vaccination against PD in different population groups, timeliness of immunization and formation of adherence to vaccination.

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# HISTORY OF MEDICINE AND ANNIVERSARIES

## TO CELEBRATE THE 500TH ANNIVERSARY OF THE BIRTH OF GABRIELE FALLOPIO (1523–1562)

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## ABSTRACT

*The article is dedicated to the 500th anniversary of the birth of the greatest physician and scientist of the 16th century Gabriele Falloppio (Fallopius), a revolutionary morphologist who made an invaluable contribution to the development of science, and one of the founders of fundamental anatomy. Although Falloppio is primarily known as an anatomist who described the uterine ("fallopian") tubes, his range of interests was much wider, and his contribution to anatomy was substantially more significant. Fallopius made many important discoveries in anatomy, and a number of anatomical structures bear his name. Also, Gabriele Falloppio was a talented teacher and a renowned medical practitioner, surgeon and pharmacist. It should be especially noted that Fallopius considered himself an apprentice of Andreas Vesalius. There is no data confirming the fact of their personal acquaintance, but there is documentary evidence of short-term correspondence between Fallopius and Vesalius. In his famous work "Anatomical observations" ("Observationes anatomicae", 1561), Fallopius pointed out Vesalius' mistakes and inaccuracies in anatomical descriptions, subjecting his "De humani corporis fabrica" to correct criticism. Vesalius' reply with compliments to Fallopius as a scientist has been preserved. In any case, the undoubted fact is that Fallopius was an adherent of Vesalius' methods in applied science and the teaching of anatomy, and consistently introduced them into practice throughout his life.*

***Key words:*** *history of anatomy, history of medicine, Gabriele Falloppio, fallopian tubes, medical terminology*

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# К 500-ЛЕТИЮ СО ДНЯ РОЖДЕНИЯ ГАБРИЭЛЕ ФАЛЛОПИО (1523–1562)

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## РЕЗЮМЕ

*Статья посвящена 500-летию со дня рождения величайшего врача и учёного XVI века Габриэле Фаллопио (Фаллопия), революционера-морфолога, внёсшего неоценимый вклад в развитие науки, одного из основателей фундаментальной анатомии. И хотя прежде всего Фаллопий известен как анатом, описавший маточные («фаллопиевы») трубы, круг интересов учёного был гораздо шире, а вклад в анатомию – несоизмеримо более значительным. Фаллопий сделал множество важных открытий в анатомии, ряд анатомических структур носят его имя. Кроме того, Габриэле Фаллопио был талантливым педагогом и известным практикующим врачом, хирургом и фармацевтом. Особо следует отметить, что Фаллопий считал себя учеником Андреаса Везалия. Данных, подтверждающих факт личного знакомства Фаллопия и Везалия, не имеется, но есть документальное подтверждение кратковременной переписки упомянутых учёных. В своём знаменитом труде «Анатомические наблюдения» («Observationes anatomicae», 1561) Фаллопий указал на ошибки Везалия и его неточности в анатомических описаниях, подвергнув корректной критике везалиевскую «De humani corporis fabrica». Сохранился ответ Везалия с комплиментами в адрес Фаллопия как учёного. В любом случае, несомненным фактом является то, что Фаллопий был приверженцем методов Везалия в прикладной науке и преподавании анатомии и последовательно внедрял их в практику на протяжении всей своей жизни.*

***Ключевые слова:*** *история анатомии, история медицины, Габриэле Фаллопио, фаллопиевы трубы, медицинская терминология*

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The year 2023 marks the 500th anniversary of the birth of Gabriele Falloppio (Latinized – Fallopius; 1523–1562) (Fig. 1), an eminent physician and morphologist, rightly considered one of the fathers of fundamental anatomy.


***FIG. 1.***
*Gabriele Fallopio of Modena (Gabriele Fallopius Mutinensis). Portrait from the library of the botanical garden of the University of Padua, Italy [1]*

For 1400 years, anatomists and surgeons, blindly following the voluminous writings of Galen (131–201), «saw what they believed» [2]. Galen did a lot of dissections and experiments, mostly on pigs and monkeys, because in those days dissection of humans was forbidden, which was the reason for the fallacy of many of his views. There were many excellent things in his writings – good descriptions of the skeletal and muscular systems, experimental studies of the function of the spinal cord by dividing it into successive levels in the pig, and so on. However, his views on blood circulation, for example, were pure conjecture, not to mention the fact that due to the lack of human material for study Galen had to extrapolate the features of the structure of animal organs to human anatomy [3]. For centuries, the study of human anatomy was reduced to the professor reading out Galen texts and students memorizing them, and the autopsy, if performed at all, was performed by an assistant (demonstrator). The professor, for example, described the kidney as lobular and the liver as consisting of five segments (as in the pig and thus as in Galen's descriptions), although both organs presented during autopsy were smooth.

At the end of the first half of the 16th century, Andreas Vesalius (1514–1564) revolutionised traditional teaching methods. He taught at Padua, where he was appointed professor of anatomy at the age of 23. Vesalius personally dissected the human body and instilled in his students the truth that it was necessary not to «see what they believe» but to «believe what they see.» One of the merits of Vesalius is that he dared to criticize the anatomical views of Galen [4-7].

After Vesalius left the chair in 1544, the place was taken by his student Matteo Realdo Colombo (1516–1559), who continued the tradition of teaching his teacher. Colombo soon moved to the chair of anatomy in Pisa and then Rome, and in Padua he was replaced by another follower of Vesalius, Gabriele Fallopio, better known by his Latinized name Fallopius. He accepted a position as professor of anatomy and surgery, and became head of the botany department (in those days, it was not uncommon for one versatilely gifted scientist to hold several positions).

Gabriele Fallopio was born in 1523 in Modena, an ancient city in Emilia-Romagna, Italy. He came from an impoverished noble family. His father Girolamo Fallopio was a jeweler by trade, but was in the military service for a number of years. It is also known that the mother of the future luminary of science was named Katerina Bergozzi. After the death of his father from syphilis, the family became impoverished, Gabriele was unable to continue his liberal arts education because of financial problems, and in 1542 he became a priest, following his uncle's example, in his parish of the Episcopal Church in Modena. However, the priesthood did not enthuse young Fallopius, although it improved his financial situation. After a few years he gave up his ministry and decided to devote himself to medicine [8, 9].

Having received an elementary liberal arts education, Fallopius became interested in anatomy, and, since there were no medical schools in Modena, he studied texts by Galen and Berengario da Carpi on his own, at the same time performing dissections of animals for educational purposes [10, 11]. In 1544, at the age of 21, Fallopius performed his first autopsy on a human cadaver under the leadership of Niccolo Macella. In those times, despite the liberalisation of the church's attitude to anatomical dissections (Pope Sixtus IV (1471–1484) was the first to allow dissection of human cadavers, namely the bodies of executed criminals, and then Pope Clement VII allowed to expand the scope of dissection possibilities), permission to dissect human beings was rarely granted to anyone, and it was necessary to have, besides a great desire to study anatomy, also influential friends to carry out one's intentions [10, 12]. Still, the 16th century was marked by the wide access of morphological scientists to dissection of the human body, which led to significant progress in the development of anatomy and medical science in general.

In 1545 Fallopius moved to Ferrara, where his teachers were Giovanni Battista Canano (1515–1579) and Antonio Musa Brassavola (1500–1555), the famous surgeon who first successfully performed tracheotomy. In parallel with his studies, Fallopius practised surgery for a time, but because of a number of lethal outcomes he abandoned the practice and resumed it only after being appointed head of the department of anatomy and surgery at Padua a few years later. In Ferrara from 1548 to 1549, Fallopius served as acting head of the department of pharmacy [9].

After Ferrara, Fallopius continued his activities at the University of Pisa, where in 1549, on the recommendation of the Duke of Florence Cosimo de' Medici, he became a lecturer and received the title of professor of anatomy [13]. Here he resumed the practice of surgery and taught anatomy until 1551. It should be noted that despite the efforts of the university administration to bring back the old ways of teaching based on Galen's texts, Fallopius taught in the progressive style of the school of Andreas Vesalius, in which he was warmly supported by the local students. The story survives of how in 1555 the university authorities attempted to revive the old style of anatomizing prescribed by statute, i.e., the junior lecturer was to read Mondino's "Anatomy" and the senior, Professor Vettor Trincavella (1490–1563), the theoretical lectures. Thus, Fallopius' role as an anatomist would have been downplayed. Trincavella's performance was eventually interrupted by students loudly chanting "vogliamo il Fallopio" ("we want Fallopio"), after which anatomy was completely in his hands [14]. Fallopius was a very bright teacher, with his character and manners he made a lasting impression on his contemporaries, all the more so when combined with an innovative approach to the educational process [3].

In parallel with teaching, Fallopius continued scientific research, dissecting with Medici's permission the corpses of lions in the Florence zoo, as a result of which he refuted Aristotle's opinion that these animals have no bone marrow [14]. While in Pisa, Fallopius conducted experiments to study the effects of opium, which was used at the time to carry out the execution of those condemned to death; as a result, the scientist was accused of vivisecting a human being (in those days, such things happened to scientists-morphologists, in particular with Vesalius).

Then Fallopius moved to Padua, where in 1551, by order of the Venetian Senate, he headed the chair of anatomy, surgery and botany at the famous university. In 1556, Fallopius was admitted to the Medical College of Venice. Throughout his career, Fallopius performed quite a few dissections of human cadavers (he was allowed to dissect up to 7-8 cadavers per year) and animals - not only adults, but also fetuses, newborns, infants, and children - which allowed him to accumulate a large information baggage on anatomy [9].

In 1561, Fallopius published his work "Anatomical Observations" ("Observationes anatomicae"). The original plan was to publish a voluminous illustrated treatise, but the scientist's friend Pietro Manna (physician to the Duke of Milan Francesco Sforza) advised him not to delay the publication of his discoveries, otherwise one of his contemporaries-colleagues would beat him to it. Thanks to this, a small book without illustrations was soon published, which later turned out to be the only one printed during Fallopius' lifetime and dedicated to his friend Pietro Manna [10, 11]. This work, in the best Vesalian tradition, describes a number of discoveries he made in human anatomy. These include a detailed description of the canal of the facial nerve («Fallopian aqueduct» or «aquaeductus Fallopii») and the chorda tympani (a branch of the facial nerve), as well as the «Fallopian hiatus» (in which the greater petrosal nerve passes), the bony labyrinth of the inner ear and, in particular, the semicircular canals and the cochlea. He first described the tympanic membrane and its relationship with surrounding bony structures. At that time two auditory ossicles were known, and Fallopius is the discoverer of the third of these, the stirrup. He also described the round and oval windows (fenestræ) and their communication with the vestibule and cochlea. Fallopius first discovered the communication between the mastoid cells and the middle ear [9]. These discoveries were realized thanks to a particularly careful method of dissection, developed by Fallopius and fundamentally different from the methods of his predecessors, who traditionally examined the skull cavity during autopsy in the last turn, which left no chance of freshness of the contents of the skull. Additionally, Fallopius used the most gentle methods of access to the base of brain and used special sharp-ground miniaturised instruments [10]. Fallopius more thoroughly than other researchers studied the ways of outflow of lachrymal fluid, described the topography of the ethmoid bone and the communication of its cells with the nasal cavity, as well as the structure of the sphenoid bone. Fallopius was also the first to describe the muscles of the soft palate, pharynx, and the pyramidal (later Fallopian) muscle [9]. He was the first to describe the anastomotic arterial ring at the base of brain, now known as the Willis' circle (polygon), in 1561, almost a century before Thomas Willis (1621–1675) [15]. Along with the above-mentioned topics, the book contains a section with a detailed description of the anatomy of female genital organs – vagina, hymen, ovaries, clitoris, and, of course, uterine tubes, which later received the name «Fallopian tubes» in honour of the scientist [6, 9]. The Russian translation of the fragment describing the fallopian tube can be found in our previous study [16]. Moreover, Fallopius described an obstetric anomaly in which the embryo implants in one of the tubes (ectopic, or tubal, pregnancy). To be fair, it should be noted that the fallopian tubes, although named after Fallopius, were described more than 1800 years earlier by Herophilus (although the latter erroneously believed that the functional role of the tubes is reduced to the transport of «female sperm» from the ovaries to the bladder). It was Fallopius who corrected this error many centuries later. But the inguinal ligament should rightfully be called the Fallopian ligament (although in a number of sources the inguinal ligaments are called «Fallopian arches»), because our hero

described it half a century before the French anatomist François Poupart (1661–1709) [12, 17, 18]. It should be especially noted that Fallopius, like Galen and Vesalius, found similarities between the male and female genital systems and noted the analogy between the clitoris and the penis, as well as common structural features of fallopian tubes and seminal ducts, but he interpreted the similarities and differences more correctly [9]. The scientist refuted the view that the ovary contains sperm and that the fallopian tubes are the functional analog of the male ejaculatory ducts; in his book in 1561, he wrote that the ovary contains oocytes that are fertilized by male sperm [13].

The interests of Fallopius covered practically all the systems of the human organism: nervous, vascular, digestive, urinary reproductive – as well as the patterns in their development. He described the ileocecal (named the Fallopian valve in his honor) valve, discovered the *valvulae conniventes* and the villi of the small intestine. He also paid much attention to the intricacies of the structure of the kidneys (first describing the tubules and calyxes), ureters, and bladder, being the first to point out the three-layered architecture of its muscular tunic [9]. Fallopius made a significant contribution to anatomical terminology: he gave scientific names (which are still preserved today) to the vagina and placenta, proposed the terms "cochlea", "labyrinth", "hard and soft palate", "tympanic membrane","cricoid cartilage". His descriptions of the trochlear, trigeminal, vestibulo-cochlear and glosso-pharyngeal nerves were the best for his time [3]. Although it was not a systematic textbook, it covered a wide range of subjects with an emphasis on the skeleton, especially the skull, and muscles. In addition to the above organs and structures, the book contains descriptions of the carotid and vertebral arteries, the muscles of the head and neck (including the muscles of the external ear and masticatory muscles), as well as the muscles of the eyeball, and some muscles of the trunk and extremities. The scientist also touched upon the peculiarities of the structure of the scalp and face [9, 14].

Fallopius laid the foundations of embryology by investigating the process of tooth development (he described the tooth premordium and the process of replacing deciduous teeth with permanent teeth) and the mechanisms of primary and secondary ossification in the sternum, skull bones, and pelvic bone [6, 9]. He tried to explain the patterns of organismal development by studying the anatomy of embryos, fetuses, children and adults, thus introducing embryology as a method of studying anatomy. This method was then refined by two of his most famous students, Hieronymus Fabricius (Fabricius ab Aquapendente or Girolamo Fabrizi d'Acquapendente, 1533–1619) and Volcher Koyter (1534–1576) [12].

It is known that Gabriele Fallopio considered himself a disciple of Vesalius. Although in reality their acquaintance was limited to a short correspondence, in February 1561 Vesalius received a copy of Fallopio's "Anatomical Observations" with additions and corrections to Vesalius's Fabrica. By the end of the year, Vesalius had compiled a response to the "Anatomicarum Gabrielis Fallopii Observum Examen", commonly referred to as Examen. Vesalius was quite upset by Fallopius's reckless arrogance to criticize him; in his letter he tried rather clumsily and unsuccessfully to refute the Italian's arguments. But despite this, he recognized Fallopius as an equal in anatomical sectional practice [7, 13]. In hard times for Andreas Vesalius, when almost everyone turned away from him, and, in particular, the mentioned Colombo became one of the most zealous critics and persecutors of the scientist, only Gabriele Fallopio not only did not deny Vesalius, but, coming to the position of his predecessor in the chair at the University of Padua Realdo Colombo, called himself a follower and disciple of Vesalius and restored his traditions in teaching and science [19].

Notwithstanding the fact that Fallopius was a zealous follower of Vesalius, in his book he noted a number of errors and inaccuracies made by his idol, but he pointed them out in a mild and friendly manner, unlike his much more rigid contemporaries, including Vesalius himself, who criticised Galen quite sharply. In particular, Fallopius clarified information about the structure and function of the round ligament of the uterus, which Vesalius mistakenly believed to be a muscle; Fallopius also determined that the inferior vena cava drains blood from the liver, not the heart, and indicated the correct direction of the outflow of bile (Vesalius believed that the bile ducts open into the stomach). Historians of medicine believe that Fallopius made more discoveries than Vesalius, and Fallopius' research is more accurate. For example, Daremberg (Daremberg C.V., 1817–1872) in his "Histoire des Sciences Medicales" (Paris: Baillie're, 1870) states, "Fallopius had the genius of invention, Vesalius the genius of method; in other words, Fallopius had genius, Vesalius had only knowledge." And if in honor of Fallopius is named many anatomical formations, the name of Vesalius - only a small non-permanent hole in the large wing of the cuneiform bone. Fallopius, however, did not publish such a voluminous work as Vesalius, and did not attach much importance to illustrations, but he was the most serious researcher of anatomy, "the great tireless inventor", as Haller (Albrecht von Haller, 1708–1777) called him, and in the polemic with Fallopius, the impartial critic Vesalius admits that his opponent was often right [5, 20].

In his book, Fallopius, following Vesalius, corrected the errors of Galen, who based most of his observations on the results of animal autopsies. This required a special courage, because in those days the ideas of Galen were dominant in anatomical science, and to contradict them was tantamount to heresy. Galen, in particular, believed that the lower jaw was made up of two bones, the sternum was made up of seven fragments, and the humerus was the second largest bone in the body after the femur. These (and a number of other) misconceptions were corrected by Fallopius [4, 9, 20].

Besides «Observationes anatomicae», following Fallopius' death, books on anatomy "Exsitio in librum Galeni de ossibus" (1570), "Observationes de venis" (1570), "De humani corporis anatome compendium" (1571) and "De partibus similaribus humani corporis" (1575), based on his lecture notes, were published [8].

A superb morphologist, Fallopio was also an equally outstanding clinician. He established the unity of the disease manifestations and refuted the hypotheses of other scientists who based its occurrence on the disorder of circulation and relationships of the main «humors» (fluids) [20]. Among the most famous studies of Fallopius is the study of the clinic, differential diagnosis and treatment of syphilis. And, it should be noted, perhaps his most famous clinical achievement was the introduction of the condom and concurrently conducted one of the first clinical trials at the time. According to the scientist himself, he tested the effectiveness of his proposed «device» - a small cloth pouch impregnated with a special composition – on 1100 men who had intimate relations with courtesans who were sick with syphilis, and then put the pouch on the organ for disinfection. As a result (according to the same Fallopius) none of the participants of the experiment were infected with the disease that was rampant at that time. The treatise «De Morbo Gallico Liber Absolutissisissimus» was published in 1564 and then repeatedly reprinted [8] (Fig. 2).

Another important contribution of Fallopius to clinical practice was the treatment of nasal polyps: he was the first physician to use an ear mirror and to use sulfuric acid to remove polyps from the external ear canal. He wrote, but did not manage to publish, a number of practical manuals: treatises on ulcers, tumors and surgery, several therapeutic treatises on baths and thermal waters, on laxatives and on the composition of medicines, and a set of commentaries on Hippocrates' "On Head Wounds". These documents were manuscripts of Fallopius' lectures. In 1575, years after the author's death, they were published in Nuremberg by Volcher Koyter [8, 9, 16, 22]. With regard to infectious diseases, Fallopius, recognizing individual differences in susceptibility, declared that for ten persons exposed to an infection there were scarcely four who would become infected; thus, his great authority contributed to lessen the terror which epidemics inspired in Italy. As a successful surgeon as well as an anatomist, Fallopio was a masterful surgeon and recommended to his students the safe use of a trocar to puncture the anterior abdominal wall for ascites: that is, in the immediate vicinity of the iliac fossa rather than in the periapical region, as was commonly practised at the time. Fallopius ligated vessels and achieved rapid wound healing by using simple medicines [13, 20].


***FIG. 2.***
*Title page of "De Morbo Gallico Liber Absolutissimus" 1566 edition (photo by Federica Viazzi) [21]*

Among other things, Gabriele Fallopio served as director of the Padua Botanical Garden, the oldest in Europe, where he conducted research concerning the use of plants in pharmaceuticals. The plant genus *Fallopia* is named in his honour [12, 22].

With all this, Fallopius was dissatisfied with the salary he received while working in Padua, and in 1561 the scholar negotiated with the University of Bologna to move there with a salary of 400 scudi, which was double his previous income. But the move never materialized due to his sudden death at the age of 39 [9]. The cause of death of the great scientist is not precisely established. It is known that from 1556 to 1557, Fallopius suffered from chronic fatigue. By then, he had developed a chronic lung infection and, as he taught anatomy mostly in winter, his health was affected [12]. Some biographical works mention a probable complication of the scientist's hand wound during the autopsy or a «prolonged lung disease» and, alternatively, pleurisy, but the most likely hypothesis is tuberculosis. The great physician and anatomist was buried in the church of St. Anthony in Padua, but later, already in the XVIII century, due to repair work, the remains of Gabriele Fallopio were moved to a nearby monastery and placed in the tomb of his friend Melchior Guilandino [12, 13] (Fig. 3).

The story of a succession of anatomy pioneers doesn't end there. One of Fallopius' students, the Dutchman Volcher Koyter, became one of the founders of comparative anatomy and embryology, continuing the research begun by Fallopius into the patterns of human and animal development. Girolamo Fabrizi (Fabricius), who succeeded his teacher as head of the department in 1562 and made invaluable contributions to anatomy and embryology, opened the first ever permanent anatomical theater in Padua. Fabricius continued the tradition of teaching and research using human autopsy and in 1574 published his discovery of the presence of valves in the veins of the extremities.


***FIG. 3.***
*Tombstone on the tomb of Gabriele Fallopio, Chapter of the Convent of the Basilica of St. Anthony, Padua [23]*

At the time, he did not realize their functional significance. However, one of his students was the young Englishman William Harvey (1578–1657), who in his later work used this discovery as an important part of his reasoning about the true nature of the blood circulation [2, 3].

**Conflict of interest**

The authors of this article declare no conflicts of interest.

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# VLADIMIR YU. MISHIN (TO THE 75TH ANNIVERSARY)

On March 19, 2024 Vladimir Yu. Mishin, Doctor of Medical Sciences, Professor, Head of the Department of Phthisiology and Pulmonology of the Russian University of Medicine, Honoured Scientist of the Russian Federation, Honoured Doctor of the Russian Federation, Academician of the Academy of Electrotechnical Sciences of the Russian Federation and member of the Public Council of the Federal Penitentiary Service of Russia, will celebrate 75 years since birth, as well as 50 years of medical, scientific and pedagogical activity and 25 years of heading the Department.

V.Yu. Mishin was born in Irkutsk and graduated from the Irkutsk State Medical Institute in 1972, specializing in sanitary-hygienic activity.

In 1972–1974 he studied in residency and in 1974–1977 in postgraduate studies at the Department of Tuberculosis of the Second Moscow State Medical Institute named after N.I. Pirogov – in postgraduate studies at the Tuberculosis Department of the Second Moscow Pirogov State Medical Institute named after N.I. Pirogov and defended his PhD thesis «Long-term follow-up of tuberculin-positive and X-ray-negative adults (clinical and radiological study)». After defending his PhD thesis, he worked as an assistant at the aforementioned department.

In 1993, he transferred to work at the Central Research Institute of Tuberculosis and defended his doctoral thesis «The course of pulmonary tuberculosis in various disorders of metabolism and functional activity of lymphocytes and monocytes of peripheral blood». After defending his doctoral thesis, he worked as the chief scientific officer of this institute.

In 1998 he was promoted to the position of Professor, and in 1999 – to the position of the Head of the Department of Phthisiology and Pulmonology at the Moscow State Medical and Dentistry University named after A.I. Evdokimov, in 2023 renamed to the Russian University of Medicine, which he heads to the present time. Also, he was Dean of the Faculty of Penitentiary Medicine from 2008 to 2018.

V. Yu. Mishin is a well-known scientist in the field of phthisiatry and pulmonology in Russia and abroad. The main directions of his scientific activity are the study of pathogenesis, drug resistance of *Mycobacterium tuberculosis*, clinic, differential diagnosis and treatment of pulmonary tuberculosis.

V.Yu. Mishin is the author of 6 patents and invention certificates, more than 900 printed scientific papers, 10 monographs, 17 chapters in monographs and manuals, 21 methodological recommendations, 25 textbooks for students and doctors, and four editions of textbooks «Phthisiopulmonology» (2000) and «Phthisiatry» (2015, 2020, 2021) for students of medical universities of the Russian Federation. He had trained 9 doctors and 34 candidates in medical sciences.

On October 7, 2022, by the Decree of the President of the Russian Federation, V.Yu. Mishin was awarded the honorary title «Honored Worker of Science of the Russian Federation».

Vladimir Yu. Mishin enjoys well-deserved authority among phthisiatricians of Russia. Colleagues and students cordially congratulate V.Yu. Mishin with his 75th anniversary and wish him further achievements in scientific, medical and pedagogical activities, health and well-being.

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