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СОДЕРЖАНИЕ

CONTENTS

Dear authors and readers,

Here is the first issue of the journal “Bulletin of Siberian Medicine” in 2023. The publication of this issue was facilitated by the great work of the editorial board and the editorial team, who made every effort to ensure that the authors could present their latest scientific achievements and familiarize readers with new developments in the field of fundamental and clinical medicine.

The year 2023 was declared the year of the teacher and mentor by the Decree of the President of Russia Vladimir Putin. It is dedicated to you – those who are actively involved in the education and upbringing of younger generation, lead the training of young highly qualified researchers, teaching staff, and medical personnel, and pass on invaluable experience and knowledge to them!

The journal invites researchers and practitioners to share up-to-date results of their research projects. We hope that you will find a lot of interesting and high-quality information in our journal and present your original ideas and technologies here. We are open to new ideas, discussions, and exchange of opinions!

We look forward to cooperating with Russian and international authors and wish the authors and readers of the journal creative and professional growth!

Looking forward to fruitful cooperation,
Professor Olga I. Urazova, Dr. Sci. (Med.),
Corresponding Member of RAS

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Predictive value of inflammatory regulators TGFb1 and CXCL8 in tumor tissue in colorectal cancer

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ABSTRACT

Background. Colorectal cancer is ranked third in terms of incidence and second in terms of mortality around the world. Molecular markers of chemoresistance allow to determine the prognosis of the disease and sensitivity of the tumor to drugs.

Aim. To assess the predictive value of expression of regulators of tumor-associated inflammation TGFb1 and CXCL8 in the tumor tissue in colorectal cancer.

Materials and methods. Patients were divided into 3 groups: group I included patients without relapse of the disease, group II encompassed patients with relapse of the disease (within 6–16 months after the end of chemotherapy), group III included patients with disease progression. Expression of TGFb1 and CXCL8 in the tumor tissue before treatment in patients with stage II–III colorectal cancer (n = 77) was determined using quantitative real-time polymerase chain reaction (PCR) on the Bio-Rad CFX-96 Touch Real-Time PCR Detection System (USA). Statistical data processing was performed using Statistica 13.0 software (StatSoft, USA).

Results. We found that in samples of poorly differentiated colorectal cancer, the level of TGFb and CXCL8 mRNA was significantly higher than in moderately and well differentiated tumors. We did not reveal any relationship of the level of TGFb1 and CXCL8 transcripts in tumor samples of patients with stage II–III colorectal cancer with age and the presence of mutations in the EGFR (Epidermal Growth Factor Receptor) signaling pathway (RAS, BRAF). We found a strong positive correlation between the levels of TGFb1 and CXCL8 transcripts for the entire sample of patients with colorectal cancer. We have found that the expression of *TGFb1* and *CXCL8* genes was significantly higher in the tumor tissue of patients with disease progression.

Conclusion. Overexpression of *TGFb1* and *CXCL8*, which are involved in the mechanism of tumor-associated inflammation, can be considered as a negative prognostic factor for the progression-free interval when using the FOLFOX / XELOX regimen for the treatment of colorectal cancer.

Keywords: colorectal cancer, CXCL8, TGFb1, EGFR, tumor progression

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Предиктивная значимость регуляторов воспаления TGFb1 и CXCL8 в опухолевой ткани при колоректальном раке

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РЕЗЮМЕ

Колоректальный рак (КРР) по заболеваемости в мире находится на 3-м месте и на 2-м – по смертности. Молекулярные маркеры химиорезистентности позволят определять прогноз заболевания и чувствительность опухоли к лекарственным препаратам.

Цель. Оценить предиктивную значимость экспрессии факторов TGFb1 и CXCL8 – регуляторов опухоль-ассоциированного воспаления в опухолевой ткани при КРР.

Материалы и методы. Пациенты были разделены на три группы: I – без рецидива, II – с рецидивом (в течение 6–16 мес после окончания химиотерапии), III – с прогрессированием заболевания. Экспрессию TGFb1 и CXCL8 в опухолевой ткани до начала лечения пациентов с КРР на II–III стадии ($n = 77$) определяли с использованием количественной полимеразной цепной реакции в реальном времени на амплификаторе CFX-96 BioRad (США). Статистическая обработка данных выполнена с использованием программного обеспечения Statistica 13.0 (StatSoft, США).

Результаты. В образцах низкодифференцированных опухолей при КРР уровень мРНК TGFb и CXCL8 был существенно выше, чем в опухолевых образцах с умеренной и высокой дифференцировкой. Зависимости уровня транскриптов TGFb1 и CXCL8 в образцах опухоли у пациентов на II–III стадии КРР от возраста и наличия мутаций EGFR (Epidermal Growth Factor Receptor) сигнального пути (RAS, BRAF) не выявлено. Установлена положительная сильная корреляционная связь между уровнями транскриптов TGFb1 и CXCL8 для всей выборки пациентов с КРР. Экспрессия генов *TGFb1* и *CXCL8* значимо выше в опухолевой ткани пациентов с прогрессированием заболевания.

Заключение. Гиперэкспрессия *TGFb1* и *CXCL8*, участвующих в механизме опухоль-ассоциированного воспаления, может рассматриваться как негативный фактор прогноза времени без прогрессирования при использовании схемы FOLFOX/XELOX лечения колоректального рака.

Ключевые слова: колоректальный рак, TGFb1, CXCL8, EGFR, опухолевая прогрессия

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Colorectal cancer (CRC) is a malignant tumor that develops in the colonic and rectal mucosa. CRC is ranked third in terms of incidence and second in terms of mortality around the world [1]. Molecular markers of chemoresistance can be used for early diagnosis of CRC, assessment of patient prognosis, and prediction of tumor sensitivity to chemotherapy. In ordinary cells, transforming growth factor beta 1 (TGFb1) stimulates production of collagen and fibronectin, reducing secretion of enzymes that are responsible for degrading the extracellular matrix [2]. At different stages of malignant transformation in colonic epithelial cells, TGFb1 acts both as a suppressor and a promoter of tumor growth [3]. TGFb1 is involved in inhibition of cell proliferation, induces apoptosis and angiogenesis, and has immunosuppressive effects [4–7]. Previous studies have shown a relationship between a high level of TGFb1 in the blood serum in patients with CRC and a poor disease prognosis [8]. TGFb1 is also involved in the epithelial – mesenchymal transition (EMT) [9–11].

It has been shown that CRC cells can produce interleukin (IL)-8 (IL-8 / CXCL8), which mediates neutrophil chemotaxis [12]. Activated neutrophils secrete CXCL8, which can interrupt the apoptotic effect of Bcl-2, prolong the presence of neutrophils in the tumor stroma, and block the anti-inflammatory effect of factors [13, 14]. CXCL8 is also involved in tumor vascularization [15].

Due to conflicting literature data on the role of inflammatory mediators in carcinogenesis, the aim of the study was to assess the predictive value of TGFb1 and CXCL8 expression in the tumor tissue in CRC.

MATERIALS AND METHODS

A retrospective study was carried out at Ulyanovsk Regional Clinical Oncology Center and Research Medical and Biological Center of Ulyanovsk State University from 2014 to 2020. The study protocol was approved by the Ethics Committee at the Institute of Medicine, Ecology, and Physical Education of Ulyanovsk State University (Protocol No. 9 of 15.09.2014).

Detailed characteristics of patients are given in Table 1.

Table 1

Characteristics of patients with colorectal cancer included in the study, *n* = 77

Parameter	Number of patients
Gender:	
– male;	42
– female	35
Age, years:	
– 25–44	9
– 45–59	40
– 60–75	28
Stage of the disease:	
– II;	16
– III;	37
– IV	24
Assessment of regional lymph node metastasis (N):	
–N0;	34
–N1;	44
–N2	16
Degree of tumor differentiation:	
– poorly differentiated;	7
– moderately differentiated;	44
– well differentiated	26
Tumor location (side):	
– left-sided	59
right-sided	18
The presence of mutations in the EGFR signaling pathway:	
– nRAS;	5
– kRAS;	21
– BRAF;	5
– undefined	12
Family history of the disease:	
– yes;	12
– no;	49
– undefined	16
Polychemotherapy according to the FOLFOX / XELOX regimen:	
– adjuvant;	53
– palliative	24
Assessment of prevalence of stage II–III primary tumor:	
– T_2	3
– T_3	34
– T_{4a}	9
– T_{4b}	7
Presence of negative prognostic factors (stage II–III tumors):	
– yes	23
– no	30

Treatment efficacy was evaluated every 2 months (after 4 courses of FOLFOX / 2 courses of XELOX), as well as after completion of all chemotherapy courses. The examination plan included: complete blood count and blood biochemistry, urinalysis, assessment of serum carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 19-9) levels, chest radiography in two projections, abdominal, pelvic, and retroperitoneal ultrasound, and endoscopic methods (fiberoptic colonoscopy, if indicated). In case of doubtful results of standard examination methods, contrast-enhanced multislice computed tomography (MSCT) or magnetic resonance imaging (MRI)

of the chest, abdomen, and pelvis was performed. At the end of treatment, patients were followed up by an oncologist, with periodic health checkups in accordance with standard WHO guidelines.

Depending on the response to FOLFOX / XELOX chemotherapy regimens, the patients were divided into 3 groups: group I included patients without relapse of the disease (more than 3 years after the end of chemotherapy), group II encompassed patients with relapse of the disease (within 6–16 months after the end of chemotherapy), group III included patients with disease progression during chemotherapy.

A molecular genetic study of formalin-fixed paraffin-embedded (FFPE) tumor samples by polymerase chain reaction (PCR) was performed in the following way. Histological sections of tumors containing at least 80% of cancer cells were used as biomaterial for the study. Sections of tissue blocks obtained from resection margins of the same tumors were taken as a conditional norm. DNA / RNA was isolated from FFPE blocks from 10–15 μm-thick tumor sections (with a total area of at least 2 cm^2) using SileksMagNA magnetic particles (Kit KIRFFPE0100, Sileks LLC, Moscow, Russia).

Using the QuantumDNA-211 kit, the concentration of DNA isolated from paraffin-embedded colorectal tissue and suitable for amplification was determined, and the presence of PCR inhibitors in the sample was identified. In 92% of cases, the samples did not contain PCR inhibitors and had a concentration of DNA fragments suitable for PCR. Furthermore, using the Insider NRAS-3 and Insider KRAS-2 Mutation Detection Kits (StepOne Plus, Evrogen Lab, Moscow, Russia), the presence of RAS mutations was determined. To analyze mutations in the *BRAF* (*V600E*) gene in tumor DNA samples, the kit manufactured by Syntol (Moscow, Russia) was used. For the transcript analysis, a reverse transcription PCR was performed immediately after the isolation. Then quantitative real-time PCR was performed in triplets on the Bio-Rad CFX-96 Touch Real-Time PCR Detection System (USA) using the DNA intercalating dye SYBR. Primer sequences synthesized at Evrogen Lab are given in Table 2. The *GAPDH* gene was used as a housekeeping gene. Normalized expression of target genes with respect to the housekeeping gene was calculated using the Bio-Rad CFX Manager Software [16].

Statistical data processing was performed using Statistica 13.0 (StatSoft, USA). Non-normally distributed variables were compared using the nonparametric Mann – Whitney test and the Pearson's correlation coefficient. To analyze regression for overall and relapse-free survival, the Cox regression model and the Kaplan – Meier analysis were used. The data were presented as the median and the interquartile range *Me* (Q_1–Q_3).

Table 2

Primer sequences in the studied genes [17]

Studied gene	Sequence	Primer annealing temperature, °C
TGFb1	F5'-CGA CTC GCC AGA GTG GTT AT -3' R 5'- AGT GAA CCC GTT GAT GTC CA-3'	59
CXCL8	F5'- CTC CAA ACC TTT CCA CCC C -3' R5'-GAT TCT TGG ATA CCA CAG AGA ATG - 3'	60
GAPDH	F5'-GCA CCG TCA AGG CTG AGA AC - 3' R5' -TGG TGA AGA CGC CAG TGG A - 3'	59

RESULTS

Following our studies, we found that the level of TGFb1 mRNA in the tumor in patients with stage II–III CRC did not depend on patient's age and the presence of mutations in the EGFR signaling pathway (RAS, BRAF). Pronounced differences in the levels of TGFb1 mRNA in CRC were detected in poorly differentiated tumors (Table 3).

A strong positive correlation was found between the levels of TGFb1 and CXCL8 transcripts in all CRC samples (r = 0.730; Rho = 0.852; p = 0.00001) (Fig. 1).

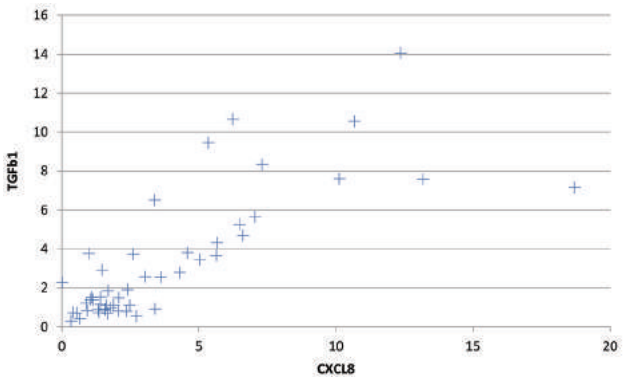

Fig. 1. Scatterplot (Pearson's correlation coefficient) of TGFb1 and CXCL8 mRNA values in the tumor in CRC patients

Table 3

Transcript level in *TGFb1* and *CXCL8* genes in FFPE colorectal cancer samples, *Me* (Q_1–Q_3)

Parameter	Normalized expression of *TGFb1* in colorectal cancer samples	Normalized expression of *CXCL8* in colorectal cancer samples
Patient's age: – over 55 years; – under 55 years	 1.845 (0.910–3.906) 2.702 (0.895–4.145) $p = 0.690$	 1.968 (1.127–5.114) 2.210 (1.549–4.997) $p = 0.560$
CRC stage: – II – III	 2.558 (1.427–7.167) 1.490 (0.867–3.769) $p = 0.114$	 2.446 (1.469–5.348) 2.212 (1.320–5.657) $p = 0.819$
Degree of tumor differentiation: – poorly differentiated; – moderately differentiated; – well differentiated	 7.168 (4.120–12.553) 2.568 (1.856–6.345) 1.427 (0.809–2.628) $p_1 = 0.035$, $p_2 = 0.023$	 8.770 (1.127–15.114) 2.262 (1.454–6.872) 1.408 (0.849–2.997) $p_1 = 0.004$, $p_2 = 0.012$
The presence of mutations in the EGFR signaling pathway (RAS, BRAF): – yes; – no	 1.630 (0.840–3.843) 2.578 (1.12–4.411) $p = 0.371$	 1.597 (1.107–3.224) 2.822 (1.647–5.294) $p = 0.246$

Note: the nonparametric Mann – Whitney test was used; the differences between two independent groups were assessed; the differences were statistically significant at $p \leq 0.05$.

It was found that the expression of TGFb1 in the tumor differed significantly in groups of patients with CRC, depending on the tumor response to standard chemotherapy. In the group of patients with disease progression during chemotherapy, the levels of TGFb1 mRNA were higher than in the group of CRC patients with relapse of the disease (within 6–16 months after the end of chemotherapy – group II) and the group of patients without relapse of the disease (for more than 2 years) – group I ($p_1 = 0.009$; $p_2 = 0.0007$). A similar trend was observed when the level of CXCL8 mRNA was analyzed (Fig. 2). Overexpression of CXCL8 in CRC was observed in the group of patients with disease progression (group III) ($p_1 = 0.0008$; $p_2 = 0.001$).

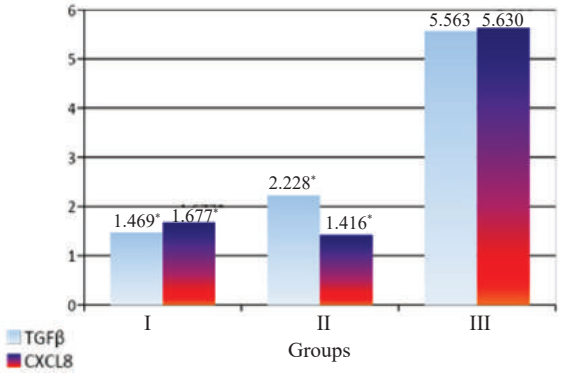

Fig. 2. TGFb1 and CXCL8 transcript levels in tumors of CRC patients depending on the tumor response to chemotherapy: * data are significantly different from those in group III ($p \leq 0.05$)

The Cox regression analysis showed that the progression-free survival depended on the expression of *TGFb1* in the primary tumor ($\chi 2 = 8.158$; $p = 0.0043$). The Kaplan – Meier analysis of relapse-free survival (PFS) in CRC patients also showed the effect of *TGFb1* expression on PFS. In the group of patients with tumor *TGFb1* expression of more than 2 (group I), the follow-up median was 11.3 months versus 62.9 months (group 0) (log-rank-test, $p = 0.041$) (Fig. 3).

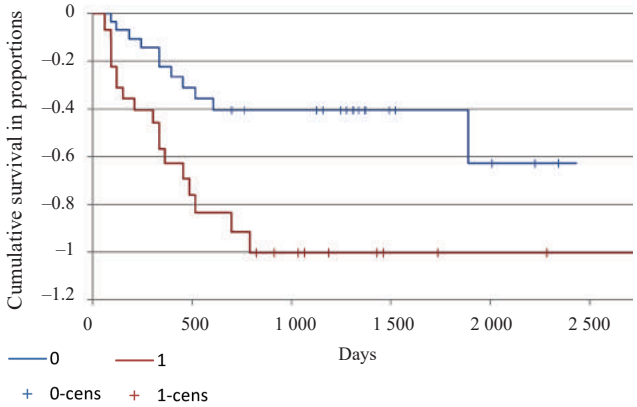

Fig. 3. Progression-free survival curve in CRC patients depending on the expression of TGFb1 in the tumor

DISCUSSION

Ambiguous functioning of TGFb1 in malignant transformation and tumor progression may be explained by the fact that, besides the two main pathways in

which TGFb is involved [18], the cytokine contributes to a number of signaling cascades, which are linked through activation of TGFb-EGFR proteins [19, 20]. During CRC progression, mutation-associated inactivation of the TGFb1 signaling pathway occurs. TGFb1 is believed to inhibit tumor growth in the intestine due to inactivation of TGF beta receptors (TGFb-R1 and R2) or intracellular SMADs (SMAD 2 / 3 / 4) [21]. Cells that lack signals from TGFb1 increase production of proinflammatory cytokines and thereby cause transformation of colonic epithelium [22, 23].

Our data on the increase in TGFb1 mRNA expression in group III with a decrease in tumor differentiation confirm the results of studies by A. Calon et al. (2012) on more frequent cancer relapses, advanced cancer stage at diagnosis, and reduced survival of patients with colon cancer [24]. The loss of the ability to suppress tumor growth (group III), which accompanies TGFb1 overexpression, determines cell selection for survival in CRC. In turn, secretion of chemokines in the tumor activates immune infiltration in the tissue and promotes migration of cancer cells to the vessels, accelerating angiogenesis. The observed coexpression of *TGFb1* and *CXCL8* genes in the CRC samples may indicate a relationship between the factors involved in the control over proliferation (TGFb1) and proinflammatory microenvironment, in particular CXCL8, during progression of CRC [24]. A shorter relapse-free interval during chemotherapy in patients with overexpression of *TGFb1* and *CXCL8* can be explained by the fact that TGFb1 protects cancer cells from apoptosis by activating the ErK signaling pathway [25].

Therefore, we found significant differences in the levels of TGFb1 and CXCL8 expression in the tumor tissue of CRC patients depending on the tumor response to chemotherapy, tumor differentiation, and the duration of the progression-free interval during FOLFOX / XELOX chemotherapy.

CONCLUSION

Overexpression of *TGFb1* and *CXCL8*, which are involved in activating the mechanisms of tumor-associated inflammation, can be considered as a negative prognostic factor for progression-free survival using the FOLFOX / XELOX treatment regimen for CRC.

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Authors contribution

Bogomolova I.A. – selection of the clinical site for the analysis. Antoneeva I.I. – conception and design. Myagdieva I.R., Abakumova T.V. – analysis and interpretation of the data. Dolgova D.R. – justification of the manuscript and critical revision of the manuscript for important intellectual content. Peskov A.B. – selection of the methods for the statistical analysis. Gening T.P – final approval of the manuscript for publication.

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Pharmacological effects of a new soluble guanylate cyclase stimulator in experimental pulmonary arterial hypertension

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ABSTRACT

Aim. To assess the effect of an indolinone derivative (2-[2-[(5RS)-5-(hydroxymethyl)-3-methyl-1,3-oxazolidine-2-yliden]-2-cyanoethylidene]-1H-indole-3(2H)-one (codename – GRS) on right ventricular contractility, endothelial vasodilator function, and histologic changes in the lungs and heart in a rat model of monocrotaline-induced pulmonary hypertension.

Materials and methods. Pulmonary arterial hypertension (PAH) was induced in Wistar rats by a single subcutaneous administration of monocrotaline at a dose of 60 mg / kg. Starting from day 15 after PAH induction, the rats received either GRS at a dose of 10 mg / kg or riociguat at a dose of 1 mg / kg orally once a day. Blood pressure in the right ventricle, right ventricular weight, endothelial vasodilator function, and the histologic structure of the lungs and heart were studied after the last administration of test substances.

Results. Twenty-eight days after monocrotaline administration, the rats developed PAH, as shown by the increase in the maximal blood pressure in the right ventricle and the right ventricular weight / total heart weight ratio. GRS after multiple administration reduced the maximal blood pressure in the right ventricle, had no significant effect on its contractility, improved endothelial vasodilator function, and normalized blood pressure. Riociguat had a hypotensive effect and did not alleviate endothelial dysfunction in experimental PAH.

Conclusion. The indolinone derivative GRS and riociguat, both soluble guanylate cyclase stimulators, lowered blood pressure in the right ventricle. GRS also alleviated endothelial dysfunction in animals with experimental PAH.

Keywords: pulmonary arterial hypertension model, soluble guanylate cyclase stimulators, indolinone derivative GRS, riociguat

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No. 5378 of 24.10.2016), IPHAR LLC (Protocol No. 113/2019 of 28.09.2021), and the Bioethics Committee at Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk NRMC (Protocol No. 185092021 of 11.10.2021).

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Фармакологические эффекты нового стимулятора растворимой гуанилатциклазы при экспериментальной легочной артериальной гипертензии

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РЕЗЮМЕ

Цель исследования – изучить влияние GRS на сократительную активность правого желудочка сердца, вазодилатирующую функцию эндотелия и гистологические изменения в легких и сердце на модели легочной гипертензии, вызванной введением монокроталина у крыс.

Материалы и методы. У самцов крыс линии Wistar воспроизводили легочную артериальную гипертензию (ЛАГ) однократным подкожным введением монокроталина в дозе 60 мг/кг. Начиная с 15-х сут после моделирования ЛАГ крысам в течение 14 сут вводили в желудок соединение GRS в дозе 10 мг/кг или риоцигуат в дозе 1 мг/кг. После последнего введения веществ измеряли давление крови в правом желудочке сердца, массу правого желудочка, оценивали вазодилатирующую функцию эндотелия и изучали гистологическое строение легких и сердца.

Результаты. Через 28 сут после введения монокроталина у крыс развивалась модель ЛАГ: повышались максимальное давление крови в правом желудочке сердца и отношение массы стенки правого желудочка к массе сердца. Соединение GRS при курсовом введении уменьшало максимальное давление крови в правом желудочке сердца, не оказывало статистически значимого влияния на его сократительную активность, улучшало вазодилатирующую функцию эндотелия, нормализовало системное артериальное давление. Риоцигуат оказывал гипотензивный эффект и не устранял дисфункцию эндотелия при экспериментальной легочной артериальной гипертензии.

Заключение. Стимуляторы растворимой гуанилатциклазы, производное индолинона GRS и риоцигуат снижают давление крови в правом желудочке сердца, соединение GRS устраняет проявления эндотелиальной дисфункции у животных с моделью ЛАГ.

Ключевые слова: модель легочной артериальной гипертензии, стимуляторы растворимой гуанилатциклазы, производное индолинона GRS, риоцигуат

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) increases the mean blood pressure in the pulmonary circulation to ≥ 25 mm Hg, causes hyperplasia of endothelium and smooth muscle cells in the pulmonary artery and perivascular inflammatory infiltrates and fibrosis in the lungs, and leads to right ventricular hypertrophy [1].

One of the causes of PAH is dysfunction of pulmonary vascular endothelium accompanied by a decrease in the production of vasodilator and antithrombotic factors, namely nitric oxide (NO) and prostacyclin [2]. NO deficiency disrupts activation of soluble guanylate cyclase (sGC) in the vascular smooth muscles and synthesis of cyclic 3',5'-guanosine monophosphate (cGMP), which is the secondary messenger [3, 4]. In patients over 18 years old, PAH and chronic thromboembolic pulmonary hypertension are treated with the NO-independent sGC stimulator riociguat. It increases sensitivity of sGC heme to low NO concentrations by stabilizing NO – sGC binding and increases cGMP production. This cyclic nucleotide activates calcium-dependent ATPase in the sarcoplasmic reticulum of the vascular smooth muscle cells, leading to subsequent deposition of calcium ions, pulmonary artery dilation, and an increase in pulmonary circulation and lung functioning [5].

The new indolinone derivative (2-[2-[(5RS)-5-(hydroxymethyl)-3-methyl-1,3-oxazolidine-2-yliden]-2-cyanoethylidene]-1H-indole-3(2H)-one (codenamed GRS) increases sGC activity independent of NO, exerts antiplatelet effects, normalizes increased blood pressure, and restores endothelial dysfunction [6, 7].

The aim of the study was to assess the effect of the indolinone derivative (2-[2-[(5RS)-5-(hydroxymethyl)-3-methyl-1,3-oxazolidine-2-yliden]-2-cyanoethylidene]-1H-indole-3(2H)-one (codename – GRS) on right ventricular contractility, endothelial vasodilator function, and histologic changes in the lungs and heart in a rat model of monocrotaline-induced pulmonary hypertension.

The study focused on the effect of GRS on blood pressure in the right ventricle, endothelial function, and histologic structure of the lung and heart in the rat model of PAH induced by the administration of monocrotaline, a pyrrolizidine alkaloid found in the Crotalaria spectabilis Roth plant. Monocrotaline pyrrole, which is its active metabolite produced in the liver, activates the extracellular calcium-sensing receptor of vascular endothelial cells, binds to DNA, inhibits cell division, and increases membrane permeability, causing pulmonary and alveolar endothelial cell apoptosis [8–10]. Riociguat was used as a reference listed drug.

MATERIALS AND METHODS

The study used 60 male Wistar rats weighing 250–320 g obtained from the Department of Experimental Biological Models of Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center of the Russian Academy of Sciences. The rats were kept in groups of 5–8 animals in standard plastic cages (VELAZ, Czech Republic) at 20–23 °C, relative humidity of no more than 50 %, exhaust – supply ratio of 8 :10, with a 12 : 12 light / dark cycle. Animal handling was performed in accordance with the European Convention for the Protection of Vertebrate Animals (Directive 2010/63/EU). The study was performed in accordance with the principles of Good Laboratory Practice and was approved by the Ethics Committee at Siberian State Medical University (Protocol No. 5378 of 24.10.2016), IPHAR LLC (Protocol No. 113/2019 of 28.09.2021), and Goldberg Research Institute of Pharmacology and Regenerative Medicine of Tomsk National Research Medical Center (Protocol No. 185092021 of 11.10.2021).

The rats were divided into 4 groups: group 1 – intact animals (n = 12), groups 2–4 – animals with PAH (n = 16 in each group); group 2 was the control group which did not receive the test substances, group 3 received GRS, group 4 received the reference listed drug riociguat (Selleckchem, USA).

PAH was simulated by a single subcutaneous administration of monocrotaline at a dose of 60 mg / kg (Sigma-Aldrich, USA). Monocrotaline was dissolved in 0.5 N HCl, then the pH was adjusted to 7.4 using 0.5 N NaOH [11].

The indolinone derivative (codenamed GRS) at a dose of 10 mg / kg and riociguat at a dose of 1 mg / kg were administered orally once a day for 14 days, starting from Day 15 after the monocrotaline administration. Pilot studies showed that the GRS dose was close to ED_{50} in terms of its antithrombotic activity [6]. The dose of riociguat (1 mg / kg) was close to its highest tolerated dose (0.03–3 mg / kg) having an antihypertensive effect. Intact and control group animals received 1 % starch solution using the same regimen as for the test substances.

On Day 28 after PAH modeling, blood pressure in the right ventricle was measured in half of the rats in each group. Then they were euthanized, so that their right ventricle weight and histologic structure of the lungs and right ventricle were studied. In the other half of the animals, we measured changes in blood pressure in response to endothelium-dependent and endothelium-independent vasodilators.

Blood pressure in the right ventricle was measured using the MP150 high-speed data acquisition system (BIOPAC Systems Inc., USA) and the TSD282 micro pressure sensor (OpSens, Canada). The rats were anesthetized by isoflurane inhalation, then the micro pressure sensor was introduced into the right ventricle of the animals through the jugular vein. The data were registered and processed using AcqKnowledge 4.2 software for the MP150 system (BIOPAC Systems Inc., USA). The maximum (P_{max}) and minimum (P_{min}) blood pressure in the right ventricle was measured in mm Hg, the maximal rate of pressure rise during one cardiac cycle (dP / dt_{max}) was measured in mm Hg / sec, the contractility index (CI) was calculated (1 / sec).

After registering the blood pressure in the right ventricle, the rats were euthanized in the carbon dioxide chamber. The heart and right ventricular wall were weighed. Right ventricular hypertrophy was calculated in mg / mg as the right ventricular wall weight / total heart weight ratio (RVWW / THW).

The functional state of endothelium was assessed as follows: the rats were anesthetized by isoflurane inhalation, then an intra-arterial catheter was implanted into the right carotid artery to measure blood pressure. Bolus doses of pharmacological agents were administered into the right femoral vein. Mean blood pressure (MBP) was registered continuously using the MP150 high-speed data acquisition system, the DA100C module, and the TSD104A sensor (AcqKnowledge 4.2.0 software, USA). Endothelium-dependent vasodilation was registered as reduction of MBP in response to intravenous acetylcholine chloride (AC) administration at a dose of 5 mcg / kg [12]. Endothelium-independent vasodilation was registered as a fall in MBP in response to sodium nitroprusside dihydrate (SN) administration at a dose of 10 mcg / kg [13]. The degree of vasodilation was determined based on the area of the triangle above the curve of MBP restoration after AC or SN administration. The short leg of the triangle represented a decrease in MBP (Δ MBP) in response to a vasodilator agent (mm Hg), while the long leg was the time of MBP restoration (sec) after the test. Endothelial dysfunction coefficient (EDC) was calculated by dividing the triangle area above the MBP restoration curve after SN administration by the triangle area after AC administration [14].

We conducted a histologic examination of deparaffinized tissue sections obtained from the lung and right ventricle stained with hematoxylin and eosin. The histologic samples were examined using the Zeiss Axio Lab.A1 microscope (Carl Zeiss AG, Germany) at 50× magnification and photographed using ZEN software (Carl Zeiss AG, Germany).

The results were statisticallyprocessed using Statistica 8.0 software (StatSoft, USA). The data were presented as $M \pm m$, where M is the mean value, m is the standard error of the mean. Multiple comparison and intergroup differences were assessed using the Kruskal – Wallis test and the Mann – Whitney test, respectively.

RESULTS AND DISCUSSION

P_{max} in the right ventricle of intact rats was 24.0 ± 2.9 mm Hg, while P_{min} was –3.3 ± 1.1 mm Hg. The values of dP / dt_{max} and CI were 53.7 ± 4.4 mm Hg / sec and 596 ± 76 1 / sec, respectively (Table 1). These values did not differ from the normal ones [15, 16].

Four weeks after monocrotaline administration (control group), P_{max} in the right ventricle increased by 1.5 times, which indicated the development of PAH, while P_{min} decreased by 1.7 times compared with blood pressure in the intact animals. The dP / dt_{max} value increased by 1.8 times ($p < 0.05$). CI was the same as in intact rats. This indicated a compensatory increase in cardiac contractions.

After continuous GRS administration, P_{max} in the right ventricle was 42.6 ± 3.1 mm Hg, which was significantly lower than in the control group ($p < 0.05$), although it did not fully improve. P_{min} was –3.4 ± 0.8 mm Hg, which was 1.6 times lower than the blood pressure in the control group and did not differ from that in the intact group.

Table 1

Effect of GRS (10 mg / kg) and riociguat (1 mg / kg) on blood pressure in the right ventricle of the heart of rats with simulated PAH, *M ± m*

Group	P_{max}, mm Hg	P_{min}, mm Hg	CI, 1 / sec	dP / dt_{max}, mm Hg / sec
Intact rats, $n = 6$	24.0 ± 2.3	–3.3 ± 1.1	53.7 ± 4.4	596 ± 76
Rats with PAH (control), $n = 8$	54.3 ± 3.2*	–5.6 ± 0.3*	45.6 ± 1.4	1, 082 ± 62*
Rats with PAH, receiving:				
– GRS, $n = 8$	42.6 ± 3.1*+	–3.4 ± 0.8+	49.6 ± 3.6	902 ± 60*
– riociguat, $n = 8$	42.1 ± 4.0*+	–3.0 ± 1.5	45.8 ± 1.5	930 ± 72*

$p < 0.05$ * compared with the intact animals; + compared with the control animals.

Differences in the values of CI and dP / dt_{max} in the control and PAH groups were not statistically significant.

After continual riociguat administration, P_{max} in the right ventricle was 1.3 times lower (42.1 ± 4.0 mm Hg) than in the control group ($p < 0.05$), although its value was significantly higher than that in the intact group. The values of dP / dt_{max} and CI did not differ from those in the rats treated only with monocrotaline. The values of P_{max}, CI, and dP / dt_{max} did not have significant differences in the GRS and riociguat groups ($p > 0.05$) (Table 1).

The indolinone derivative GRS and riociguat were equally effective in lowering P_{max} in the right ventricle of the rats with simulated PAH. The GRS compound also normalized P_{min}.

In the intact animals, the RVWW / THW ratio was 0.222 ± 0.006 mg / kg; in the rats with PAH, it increased by 1.5 times ($p < 0.05$). These changes indicated right ventricular hypertrophy, which developed due to increased blood pressure in the pulmonary circulation. The RVWW / THW ratio was still increased in the GRS and riociguat groups ($p > 0.05$) (Table 2).

Table 2

Effect of GRS (10 mg / kg) and riociguat (1 mg / kg) on the right ventricular weight to total heart weight ratio in rats with simulated PAH, *M ± m*

Group	RVWW / THW, mg / mg
Intact rats, $n = 6$	0.222 ± 0.006
Rats with PAH (control), $n = 8$	0.332 ± 0.013*
Rats with PAH, receiving:	
GRS, $n = 8$	0.306 ± 0.020*
riociguat, $n = 8$	0.314 ± 0.020*

* $p < 0.05$ compared with the intact animals.

After AC administration, MBP decreased from 111 ± 4 to 42 ± 2 mm Hg in the intact rats, and from 92 ± 3 to 34 ± 2 mm Hg in the rats with PAH. These changes indicated a weakened response of blood vessels to endothelium-dependent vasodilator AC ($p < 0.05$) (Table 3).

Table 3

Effects of GRS (10 mg / kg) and riociguat (1 mg / kg) on endothelial vasodilator function in the rats with simulated PAH, *M ± m*

Group	Acetylcholine chloride, 5 mcg / kg			
	MBP, mm Hg	Δ MBP, mm Hg	Time of MBP restoration, sec	Area of the triangle above the MBP restoration curve, mm Hg / sec
Intact rats, $n = 6$	111 ± 4	42 ± 2	56 ± 11	1,121 ± 151
Rats with PAH (control), $n = 8$	92 ± 3*	34 ± 2*	58 ± 6	993 ± 115
Rats with PAH, receiving:				
– GRS, $n = 8$	102 ± 5	41 ± 3#	73 ± 5*	1,489 ± 151+
– riociguat, $n = 8$	87 ± 3*	29 ± 2*	98 ± 13+	1,467 ± 228

Here and in Table 4: $p < 0.05$ compared with: * the intact animals; + the control animals; # the animals receiving riociguat.

Most studies observed MBP reduction in systemic circulation in monocrotaline-induced PAH [17]. This effect is caused by a decrease in cardiac output and hypoxemia, leading to vasodilation [18]. PAH also reduces the activity of angiotensin-converting enzyme in the lungs, disrupting the production of angiotensin II and weakening its vasoconstrictive effect [19]. The reduced MBP response to AC in the rats with PAH confirms vascular endothelial dysfunction.

MBP and Δ MBP in the GRS group were higher than in the group with PAH and were similar to those in the intact group. Riociguat administration did not affect MBP and ΔMBP, which remained the same as in the group with PAH. The effect of GRS on Δ MBP

after AC administration was more pronounced than that of riociguat, which is probably associated with its ability to increase NO production in the endothelium.

Riociguat considerably increased the time of MBP restoration both after AC and SN administration. The GRS compound delayed MBP restoration after AC administration, but to a lesser extent than riociguat. We assume that riociguat stimulates sGC more, binds more strongly to enzyme molecules or stabilizes sGC – NO binding more [20]. The protective effect of GRS on the endothelium was shown by a larger area of the triangle above the MBP restoration curve compared with its area after riociguat administration ($p < 0.05$).

After SN administration, MBP in the intact animals decreased from 114 ± 4 to 51 ± 2 mm Hg. In the PAH animals, the MBP response to SN was weaker ($p < 0.05$) (Table 4), indicating a decrease in sGC sensitivity to the NO effect [20].

Table 4

Effects of GRS (10 mg / kg) and riociguat (1 mg / kg) on endothelial dysfunction in the rats with simulated PAH, *M* ± *m*

Group	Sodium nitroprusside dihydrate, 10 mcg / kg				EDC
	MBP, mm Hg	Δ MBP, mm Hg	Time of MBP restoration, sec	Area of the triangle above the MBP restoration curve, mm Hg / sec	
Intact rats, $n = 6$	114 ± 4	51 ± 2	80 ± 11	2,000 ± 244	1.80 ± 0.06
Rats with PAH (control), $n = 8$	97 ± 2*	44 ± 1*	99 ± 7	2,169 ± 158	2.36 ± 0.27
Rats with PAH, receiving:					
GRS, $n = 8$;	103 ± 5	45 ± 4#	105 ± 10#	2,388 ± 340	1.59 ± 0.13+
riociguat, $n = 8$	88 ± 4*+	31 ± 2*+	178 ± 15*+	2,833 ± 398	2.20 ± 0.37

The indolinone derivative GRS after continual administration at a dose of 10 mg / kg to rats with PAH did not reduce MBP to a level lower than the value in the intact animals. In the GRS group, Δ MBP did not improve after SN administration, but it did not become less than in the intact animals. The GRS compound has an antihypertensive effect, while it does not reduce normal blood pressure and maintains its regulation by activating the oxidized form of sGC. EDC in the GRS group was lower than in the control group (Table 4).

As a hypotensive agent [5], riociguat reduced MBP and Δ MBP in response to SN administration ($p < 0.05$). It is possible that in conditions of hypoxemia and endothelial dysfunction, some sGC molecules have lost heme and become oxidized, and riociguat does not stimulate oxidized sGC molecules [20].

The histologic examination showed that interalveolar septa became significantly thicker and were sclerotized in the lungs of animals with PAH. The alveoli were deformed, alveolar type II cells and smooth muscles proliferated. Granulation tissue grew in the alveolar lumen. Endothelial cell hyperplasia and smooth muscle hypertrophy were observed in the pulmonary arteries. Such pathological changes in the lung tissue correspond to interstitial pneumonia (Fig. 1). Focal cardiomyocyte hypertrophy and interstitial myocarditis developed in the myocardium of the right ventricle (Fig. 2).

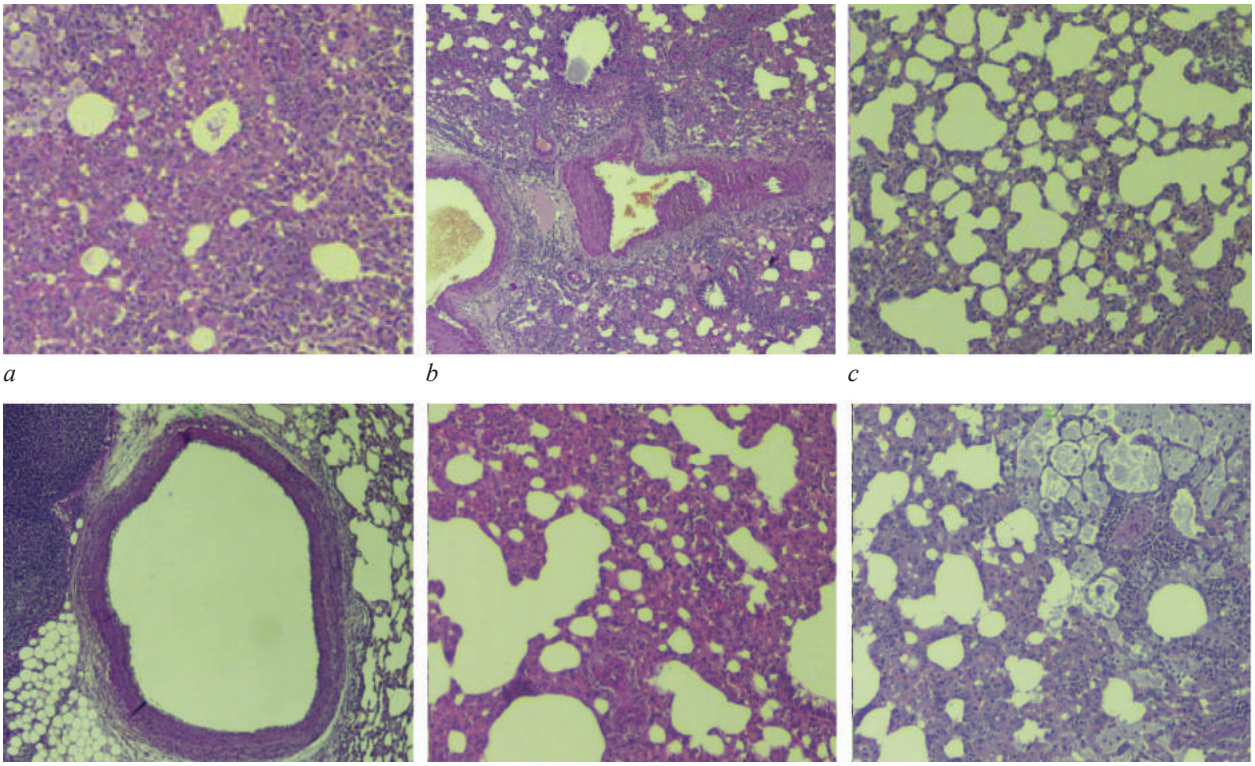
Administration of GRS and riociguat to the rats with PAH considerably decreased the thickness of the interalveolar septum; the alveoli became more open and air-filled. Alveolar type 2 cell and smooth muscle cell proliferation was less pronounced in the alveoli. Endothelial and arterial smooth muscle cells did not proliferate (Fig. 1). GRS administration reduced inflammatory infiltration in the myocardium, but cardiomyocyte hypertrophy persisted. Riociguat did not affect the myocardial pathology in PAH (Fig. 2).

CONCLUSION

The need for effective PAH treatment remains urgent [21]. Currently used drugs, such as endothelin receptor antagonists, calcium channel blockers, and iloprost, which is a prostacyclin analog, do not protect endothelium, may lower systemic blood pressure, and induce bleeding and other adverse effects. The SGC stimulator riociguat is the treatment standard for PAH, but it does not alleviate endothelial dysfunction and can cause tachycardia, arterial hypotension, and anemia [22, 23].

The new antithrombotic drug GRS, which is an indolinone derivative and a sGC stimulator, is as potent in lowering blood pressure in the right ventricle as riociguat in experimental PAH; unlike riociguat, it can also alleviate endothelial dysfunction. GRS also prevents pathological remodeling of pulmonary vessels.

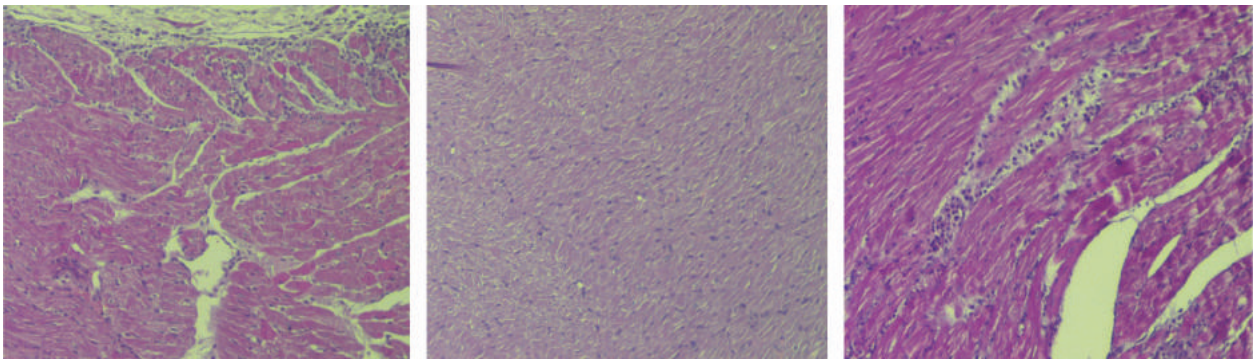
The data obtained in this study indicate the prospects of using the new antithrombotic drug, the indolinone derivative GRS, for the prevention and treatment of pulmonary arterial hypertension.



a *b* *c*

d *e* *f*

Fig. 1. Histologic changes in the lungs of the rats with simulated PAH (*a, b*) and administration of GRS at a dose of 10 mg / kg (*c, d*) and riociguat at a dose of 1 mg / kg (*e, f*). Here and in Fig. 2: staining with hematoxylin and eosin, 50× magnification



a *b* *c*

Fig. 2. Histologic changes in the right ventricular myocardium of the rats with PAH *(a)*, and administration of GRS at a dose of 10 mg / kg (*b*) and riociguat at a dose of 1 mg / kg (*c*)

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Authors contribution

Bykov V.V., Bykova A.V. – conception and design. Aliev O.I., Sidikhmenova A.V., Dunaeva O.I. – carrying out of the experiment, analysis and interpretation of the data. Khazanov V.A., Stankevich S.A. – justification of the manuscript, critical revision of the manuscript for important intellectual content. Vengerovskii A.I., Udut V.V. – final approval of the manuscript for publication.

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An extract from the culture of a thermophilic *Staphylococcus aureus* strain suppresses allergic inflammation in the airways *in vivo* and degranulation of mast cells and basophils *in vitro*

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ABSTRACT

Aim. To study the anti-allergic effects of ruzam, an extract from the culture of a thermophilic *Staphylococcus aureus* strain, in an *in vivo* model of asthma and its influence on degranulation of mast cells and basophils *in vitro*.

Materials and methods. Allergic asthma in guinea pigs was reproduced by two intraperitoneal injections of ovalbumin followed by a series of inhalations of this antigen for 1.5 months. Ruzam (6 μg / kg) or a reference drug (sodium cromoglycate, 3 mg / kg) was administered daily via a nebulizer during the last 6 days of immunization. One day after completion of inhalations with ovalbumin and compared drugs, changes in the airways were assessed using cytological, morphometric, and histologic methods. Rabbit blood basophils and rat peritoneal mast cells were used to determine the effect of ruzam on IgE-independent degranulation induced by the compound 48 / 80 *in vitro*. The effect of ruzam was compared with that of hydrocortisone hemisuccinate. Basophils from the blood of ovalbumin-sensitized guinea pigs were used to evaluate the effect of the drug on IgE-dependent degranulation induced by ovalbumin. Granules of mast cells and basophils were detected by alcian blue staining to calculate the degranulation index.

Results. In the asthma model, ruzam reduced the degree of airway obstruction by increasing the bronchoalveolar lavage volume returned and suppressed neutrophilic and eosinophilic inflammation, while mobilizing other effector cells of the anti-pathogen immunity (lymphocytes and macrophages). Ruzam has proven to have a stronger anti-allergic effect than sodium cromoglycate by several parameters. At concentrations of 8.4–840 μg / ml, ruzam inhibited degranulation of mast cells and basophils, induced by the compound 48 / 80, equally to hydrocortisone hemisuccinate (10^{-3} M). At concentrations of 280 and 420 μg / ml, ruzam dose-dependently inhibited ovalbumin-induced degranulation of basophils in sensitized guinea pigs.

Conclusion. The anti-allergic effect of ruzam was confirmed in test systems *in vivo* and *in vitro*. We speculate here that the TLR2 signaling pathway may be involved in biological and pharmacological effects of this drug.

Keywords: ruzam, *Staphylococcus aureus*, thermophilic strain, asthma model, allergic inflammation, mast cells, basophils, degranulation

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Экстракт из культуры термофильного штамма *Staphylococcus aureus* подавляет аллергическое воспаление в дыхательных путях *in vivo* и дегрануляцию тучных клеток и базофилов *in vitro*

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РЕЗЮМЕ

Цель – изучить противоаллергическое действие рузама – экстракта из культуры термофильного штамма *Staphylococcus aureus* – на модели астмы *in vivo*, а также его влияние на дегрануляцию тучных клеток и базофилов *in vitro*.

Материалы и методы. Аллергическую астму у морских свинок воспроизводили двумя внутрибрюшинными инъекциями овальбумина с последующей серией ингаляций этого антигена в течение 1,5 мес. Рузам (6 мкг/кг) или референс-препарат (кромогликат натрия, 3 мг/кг) вводили ежедневно с помощью небулайзера в течение последних 6 сут иммунизации. Через 1 сут после завершения ингаляций овальбумина и сравниваемых препаратов оценивали изменения в дыхательных путях с помощью цитологических, морфометрических и гистологических методов. Для определения влияния рузама на IgE-независимую дегрануляцию, индуцированную соединением 48/80 *in vitro*, использовали базофилы крови кроликов и перитонеальные тучные клетки крыс. Эффект рузама сравнивали с таковым гидрокортизона гемисукцината. Базофилы крови сенсибилизированных овальбумином морских свинок использовали при оценке действия препарата на IgE-зависимую дегрануляцию, индуцированную овальбумином. Гранулы тучных клеток и базофилов для расчета индекса дегрануляции окрашивали с помощью альцианового синего.

Результаты. На модели астмы рузам снижал степень обструкции дыхательных путей, повышая объем возврата бронхоальвеолярного смыва, и подавлял нейтрофильное и эозинофильное воспаление, при этом мобилизуя другие клетки-эффекторы противоинфекционного ответа (лимфоциты и макрофаги). По ряду критериев противоаллергической эффективности рузам превосходил кромогликат натрия. Рузам в концентрациях 8,4–840 мкг/мл ингибировал дегрануляцию тучных клеток и базофилов, вызванную соединением 48/80, в той же степени, что и гидрокортизона гемисукцинат (10^{-3} М). Рузам (280 и 420 мкг/мл) дозозависимо подавлял индуцированную овальбумином дегрануляцию базофилов сенсибилизированных морских свинок.

Заключение. Подтверждено противоаллергическое действие рузама в тест-системах *in vivo* и *in vitro*. Выдвинута гипотеза о TLR2-опосредованном характере биологических/фармакологических эффектов препарата.

Ключевые слова: рузам, *Staphylococcus aureus*, термофильный штамм, модель астмы, аллергическое воспаление, тучные клетки, базофилы, дегрануляция

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Исследование одобрено локальным биоэтическим комитетом ВНЦ БАВ (протокол №12 от 28.03.2006).

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INTRODUCTION

Structural components and products of microorganisms, primarily bacteria, have long been investigated to find bioactive substances with a potentially wide spectrum of pharmacological effects [1]. In this regard, development of bacterial immunomodulators is one of the most promising research areas; its viability has been proven in clinical trials and practical healthcare. [2, 3]. Ruzam, developed by a group of Russian scientists, belongs to this class of drugs. It is an extract from the culture of a thermophilic *Staphylococcus aureus* strain. Ruzam has been successfully used in the treatment of allergy for three decades [4, 5]. The effectiveness of this drug in the treatment of pollinosis, bronchial asthma, allergic rhinitis, atopic dermatitis, urticaria and angioedema, and latex, food, and insect allergies, as well as in the prevention of respiratory infections has been shown [4].

Currently, ruzam is approved for clinical use in the form of a solution for subcutaneous injections. Successful results of preclinical and clinical trials of nasal and orally inhaled forms of ruzam in the treatment of allergic respiratory diseases are quite encouraging [6].

Previous studies on the anti-allergic effect of ruzam showed that the drug is capable of suppressing ovalbumin-induced inflammation in sensitized animals *in vivo* and inhibiting IgE-dependent and IgE-independent degranulation of mast cells and basophils in test systems *in vitro.* The materials of this study were presented by A.G. Chuchalin et al. back in 2003, but only as a fragment of the review article. [5]. These data are of great interest in the context of development and implementation of new dosage forms of ruzam. Besides, they explain the pharmacological effects of the drug identified in clinical trials [4]. The above encouraged to describe in detail the results of earlier experiments and subject them to additional mathematical processing and interpretation in the light of emerging immunological paradigms.

MATERIALS AND METHODS

Ruzam is a complex of lipoproteins derived from a culture of the thermophilic *Staphylococcus aureus* strain C2. The drug was provided for testing by Ruzam-M (Russia). Allergic respiratory inflammation was reproduced *in vivo* according to the method proposed by P.A. Hutson et al. [7] and modified as described previously [8]. Ovalbumin (Sigma-Aldrich, USA) at a dose of 10 mg / kg was intraperitoneally administered twice with a 7-day interval to male and female guinea pigs weighing 300–400 g. Then the animals inhaled (Pari LC Plus nebulizer (Pari GmbH, Germany)) 1 ml of the ovalbumin solution once every 4 days for 1.5 months. The concentration of ovalbumin was gradually raised from 0.1 to 1%. Ruzam at a dose of 6 μg / kg or sodium cromoglycate at a dose of 3 mg / kg as a reference listed drug were administered via the same nebulizer in the form of an aqueous solution at a dose of 1 ml daily during the last 6 days of immunization in the animal groups “Ovalbumin + ruzam” and “Ovalbumin + cromoglycate”, respectively. In the “Ovalbumin” group, sterile water was inhaled via the nebulizer in the last days of immunization in the same mode and volume. Each inhalation lasted 180 seconds.

Aerosol particles characteristics were evaluated using the aerodynamic particle sizer APS 3300 (TSI, USA) and the cascade impactor with a subsequent fluorometric analysis of the selected samples. The average volumetric flow rate was 0.46 ml / min, the mass median aerodynamic diameter was 6.2 μm, the respirable fraction of ruzam or sodium cromoglycate was 38.5 and 39.8%, respectively. Twenty-four hours after the inhalation of the last dose of ovalbumin, changes in the

airways were assessed using cytological, morphometric, and histologic methods. Bronchoalveolar lavage fluid (BALF) was collected under hexenal anesthesia injected intraperitoneally through the endotracheal cannula by double instillation of 10 ml sterile 0.9% NaCl solution heated up to 37 °C into the lungs.

Then the BALF volume returned was evaluated. The absolute number of cells per 1 ml (cytosis) in the BALF after centrifugation at 200 g for 10 min was determined. The number of neutrophils, eosinophils, macrophages, and lymphocytes was counted in Romanowsky-stained smears [9]. The density of bronchus-associated lymphoid follicles (whitish plaques with the diameter of 3–5 mm protruding above the bronchial mucosa) was assessed in the macroslides under the magnifying glass using the ocular measuring grid proposed by G.G. Avtandilov [8].

For histologic studies, lung tissues were fixed by Carnoy's solution and embedded in paraffin. The 4–5-μm sections were stained with hematoxylin and eosin to identify eosinophils, neutrophils, macrophages, lymphocytes, and histiocytes, or with toluidine blue (pH 2.0) to determine the number of mast cells. In both cases, cells were counted at 400x magnification. Mast cell degranulation was assessed in points: 1 point – the entire cytoplasm was densely filled with dark purple granules, 2 points – vacant areas in the cytoplasm were noted; sometimes separate granules were located near the cell, 3 points – vacant areas accounted for 50–70% of the cytoplasm, the nucleus was bare, the granules were located loosely; 4 points – vacant areas accounted for more than 70% of the cytoplasm (granulolysis). The degranulation index (DI) was calculated by the formula:

$$DI = \Sigma (i \times n_i) / \Sigma n_i,$$

where i is the degree of degranulation in points, and n_i is the number of cells with i degree (%) [10].

Peritoneal mast cells from Wistar rats were obtained as described previously [11].

Basophils were isolated as part of a leukocyte suspension from the blood taken from the heart of rabbits or guinea pigs by two-stage sedimentation. At the first stage, the blood was diluted with ethylenediaminetetraacetic acid (Sigma-Aldrich, USA); at the second stage, it was diluted with a citrate-containing liquid [12]. The effect of ruzam on IgE-independent degranulation of mast cells and basophils *in vitro* was evaluated at final concentrations of 8.4, 84, and 840 μg / ml. Hydrocortisone 21-hemisuccinate sodium salt (Sigma-Aldrich, USA) at concentrations of 10^{-5}, 10^{-4}, and 10^{-3} M was used as a reference listed drug. Degranulation was induced by the compound 48 / 80 (Sigma-Aldrich, USA) (1 μg / ml), which is a polymer that causes degranulation of mast cells and basophils and liberates histamine [13].

The effect of ruzam on IgE-dependent degranulation of basophils *in vitro* was assessed as described previously [12]. The leukocyte suspension (10^4 cells / ml) was isolated from the heart blood of guinea pigs, which 1 month before were sensitized by the intraperitoneal injection of 10 μg of ovalbumin (Sigma-Aldrich, USA) with the aluminum hydroxide adjuvant (100 mg of gel) according to the method [14]. The suspension of leukocytes was incubated in a medium with ruzam (280 and 420 μg / ml) or without the drug (control) for 15 minutes at 37°C and 5% CO_2. Then a solution of ovalbumin at a final concentration of 0.35% was added to the culture for 10 minutes to induce degranulation of basophils. The reaction was stopped by adding cooled salt solution. This cell suspension was centrifuged at 100 g for 7 min. Slides for microscopy were prepared from the sediment. They were fixed and stained according to the method [15]. To detect basophil granules, 0.5% alcian blue (pH 1.0) was used. The nuclei were stained with safranin O 0.1% solution in 1% acetic acid. DI was calculated according to the same method that was used in the histologic studies [10].

Treatment and control groups in *in vivo* studies included 5 guinea pigs each. In *in vitro* studies, cell culture triplets from each of the 3 animals were used for each concentration of ruzam and the reference listed drug as well as for controls.

Statistical data processing was performed using Statistica 18 (StatSoft Inc., USA). Independent and dependent samples were compared by quantitative characteristics using the Mann – Whitney and Wilcoxon tests, respectively. Quantitative data in tables and figures were presented as the mean and the standard deviation $M \pm SE$. The differences were considered statistically significant at $p < 0.05$. A trend toward statistical significance was noted at $0.05 \leq p < 0.1$.

RESULTS

Anti-allergic effects of ruzam in the model of ovalbumin-induced airway inflammation in guinea pigs

Two intraperitoneal injections of ovalbumin, and then a series of inhalations of this antigen to guinea pigs reproduced chronic allergic airway inflammation. In addition to morphological disorders, this inflammation

was characterized by a decrease in the BALF volume returned and an increase in the number of cells in the BALF by more than 1.5 times, mainly due to eosinophils and to a lesser extent due to neutrophils (Table 1). The number and proportion of lymphocytes did not change significantly. The absolute and relative number of macrophages decreased in comparison with the intact animals.

Table 1

The effect of ruzam and sodium cromoglycate on some parameters of bronchoalveolar lavage fluid in guinea pigs with ovalbumin-induced allergic airway inflammation, *M* ± *SE*

Parameter		Intact animals	Allergic airway inflammation		
			Ovalbumin	Ovalbumin + ruzam	Ovalbumin + cromoglycate
BALF volume returned, %		65.6 ± 6.3	50.0 ± 5.8*	62.9 ± 1.0#	50.7 ± 7.4
Cytosis, cells / ml		42.0 ± 9.0	68.0 ± 16.0*	58.0 ± 7.0	55.0 ± 19.0
Macrophages	cells / ml	32.0 ± 2.9	22.2 ± 2.4*	29.6 ± 2.4†	12.6 ± 3.0*
	%	76.6 ± 5.9	32.7 ± 3.5*	51.1 ± 5.2†	22.9 ± 5.5*
Lymphocytes	cells / ml	3.3 ± 0.4	4.3 ± 1.4	6.0 ± 1.3*†	2.7 ± 0.6
	%	7.8 ± 1.0	6.4 ± 1.7	10.4 ± 2.2*†	4.9 ± 1.1
Neutrophils	cells / ml	0.30 ± 0.05	1.8 ± 0.9*	0.6 ± 0.3#†	1.5 ± 0.5
	%	0.64 ± 0.13	2.7 ± 1.4*	1.0 ± 0.5#†	2.7 ± 0.1
Eosinophils	cells / ml	6.2 ± 2.0	39.5 ± 3.3*	21.6 ± 2.9*#†	38.4 ± 3.6*
	%	14.9 ± 5.0	58.1 ± 4.8*	37.7 ± 5.1*#†	69.8 ± 6.6*

* $p < 0.05$ compared with the intact animals; # $p < 0.05$ compared with the "Ovalbumin" group; † $p < 0.05$ compared with the "Ovalbumin + cromoglycate" group

Daily ruzam inhalations for 6 days at the final stage of allergic inflammation modeling increased the BALF volume returned. This parameter was equal to that in the intact guinea pigs (Table 1). Sodium cromoglycate did not change the BALF volume returned.

A trend toward a decrease in cytosis in BALF was revealed in both groups of animals treated with either ruzam or sodium cromoglycate. However, the number of eosinophils and neutrophils reduced significantly only in the "Ovalbumin + ruzam" group.

Sodium cromoglycate contributed to an even greater decrease in the number of macrophages in BALF, whereas with ruzam inhalations, the number of these cells rose. As a result, this parameter in the "Ovalbumin + ruzam" group did not differ from that in the intact animals, but was more than twice higher than in the "Ovalbumin + cromoglycate" group (Table 1).

The number of lymphocytes in BALF tended to increase with ruzam and to decrease with sodium cromoglycate. As a result, the absolute and relative number of lymphocytes in the animals receiving ruzam was about twice as high as in the guinea pigs after reference drug inhalation.

Immunization of the guinea pigs with ovalbumin led to significant morphological changes in the airways, a combination of which can be characterized as bronchitis and obstructive emphysema. Dystrophic epithelial changes and extensive areas of desquamation were detected. In the bronchial lumen, in addition to desquamated epithelial cells, large numbers of eosinophils and neutrophils and single macrophages were present. Diffuse focal polymorphonuclear leukocyte (eosinophil and neutrophil), lymphocyte, and histiocyte infiltration was noted in the interalveolar septa. The number of polymorphonuclear leukocytes per field of view increased more than two-fold in comparison with the intact animals (Fig. 1). A statistically unconfirmed trend toward a decrease in the number of mast cells per field of view was revealed; degranulation parameters in these cells did not change significantly. The density of bronchus-associated lymphoid tissue increased approximately two-fold (Fig. 2).

The tested and reference listed drugs in the model of allergic airway inflammation approximately equally reduced eosinophil and neutrophil infiltration in the lungs: following ruzam inhalation, the number of polymorphonuclear leukocytes in the interalveolar septa decreased by 3.6 times, and after sodium cromoglycate – by 3 times (Fig. 1). No significant differences between the groups of guinea pigs receiving the compared drugs were revealed for this parameter: in both cases, the number of polymorphonuclear leukocytes decreased to the same level as in the intact animals.

Inhalations with ruzam or sodium cromoglycate did not significantly change the number of mast cells and their degranulation parameters in the lungs of the ovalbumin-sensitized guinea pigs. Besides, neither of them had any effect on the density of bronchus-associated lymphoid follicles (Fig. 2).

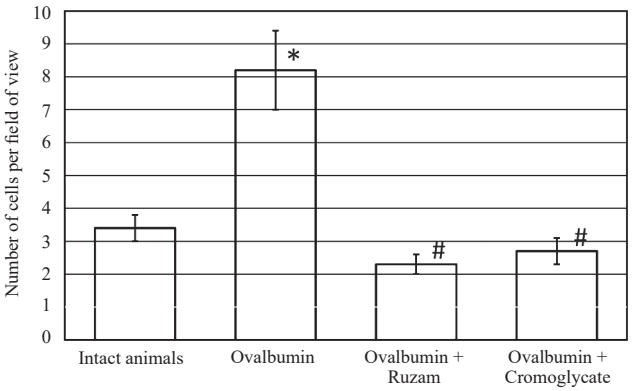

Fig. 1. The effects of ruzam and sodium cromoglycate on the number of polymorphonuclear leukocytes in the interalveolar septa in the guinea pigs with ovalbumin-induced allergic airway inflammation, $M \pm SE$: * $p < 0.05$ compared with the intact animals; # $p < 0.05$ compared with the "Ovalbumin" group

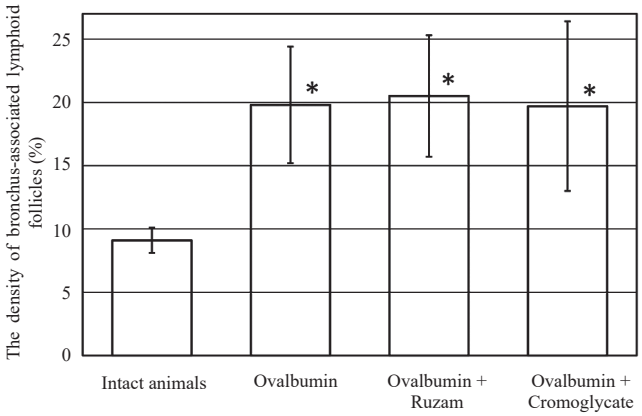

Fig. 2. The effects of ruzam and sodium cromoglycate on the density of bronchus-associated lymphoid follicles in the ovalbumin-sensitized guinea pigs, $M \pm SE$: * $p < 0.05$ compared with the intact animals

The effect of ruzam on mast cell and basophil degranulation in vitro

The compound 48 / 80 in the test systems *in vitro* quite expectedly increased DI in rat peritoneal mast cells by 6 times and in rabbit blood basophils by 2 times in comparison with that in the intact cells (Table 2).

Ruzam in a wide range of concentrations (8.4 – 840 μg / ml) reduced the index of 48 / 80-induced mast cell degranulation in rats more than two-fold (Table 2). However, the effect has not proven to be dose dependent. The reference listed drug, hydrocortisone 21-hemisuccinate, has shown to inhibit degranulation only at the highest concentrations used (10^{-3} M).

Similar patterns were revealed when evaluating the effect of ruzam and the reference listed drug on degranulation of rabbit blood basophils induced by the histamine liberator (Table 2). Ruzam at all the concentrations used reduced DI by 1.5 times, while hydrocortisone 21-hemisuccinate demonstrated a comparable effect only at the concentration of 10^{-3} M.

The addition of ovalbumin into the culture of basophils obtained from the blood of guinea pigs previously immunized with this antigen doubled DI in these cells compared with that in unstimulated basophil cultures that served as controls in this test system (Fig. 3).

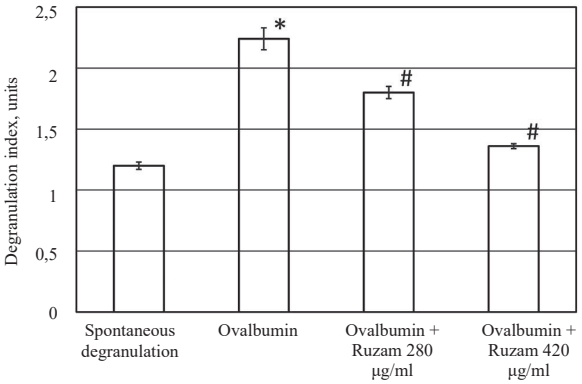

Fig. 3. The effect of ruzam on ovalbumin-induced degranulation of basophils in the ovalbumin-sensitized guinea pigs *in vitro*, $M \pm SE$: * $p < 0.01$ compared with spontaneous degranulation; # $p < 0.01$ compared with ovalbumin-induced degranulation without additional effects

Table 2

The effects of ruzam and hydrocortisone 21-hemisuccinate on *in vitro* degranulation of mast cells and basophils induced by the compound 48 / 80, $M \pm SE$

Options for influencing the cultured cells			Degranulation index, units	
			Rat peritoneal mast cells	Rabbit blood basophils
Intact cells			0.40 ± 0.05	0.70 ± 0.05
Cells stimulated by the compound 48/80	No additional effects		2.40 ± 0.10*	1.40 ± 0.10*
	Ruzam	840 μg / ml	1.10 ± 0.10*#	0.90 ± 0.05#
		84 μg / ml	1.10 ± 0.10*#	1.00 ± 0.05
		8.4 μg / ml	1.12 ± 0.10*#	0.95 ± 0.05
	Hydrocortisone 21-hemisuccinate	10^{-3} M	1.10 ± 0.05*#	0.80 ± 0.05#
		10^{-4} M	1.28 ± 0.05*	1.25 ± 0.05*
		10^{-5} M	1.50 ± 0.05*	1.65 ± 0.05*

* $p < 0.01$ compared with the intact cells; # $p < 0.01$ compared with the cells stimulated by the compound 48 / 80 without additional effects

Ruzam dose-dependently suppressed ovalbumin-induced degranulation of basophils in the sensitized animals. At the same time, at both concentrations used (280 and 420 μg / ml), the drug reduced DI to a level that was not significantly different from the control values.

DISCUSSION

The morphological and cytological changes in the model of allergic airway inflammation observed in our study are generally consistent with the results of similar studies in ovalbumin-induced asthma models [16, 17]. At the same time, when determining the number of mast cells in the lung tissue and their DI, we did not reveal significant differences compared with the intact animals. We even revealed a slight trend toward a decrease in the number of these cells, which partly contradicts previously published data on expansion of mast cells in the peribronchial tissue in patients with asthma [18] and animals with chronic allergic airway inflammation induced by ovalbumin [16].

Despite the fact that mature mast cells reside in tissues for a long time and are able to withstand repeated cycles of degranulation [19], some authors point out the possibility of temporary depletion of the population of these cells following intense degranulation [20, 21]. We conducted a histologic study of the lungs 24 hours after inhalation of the last challenging dose of ovalbumin, which *a priori* should cause massive and rapid degranulation of mast cells in the airways. This, in turn, could lead to a short-term decrease in the number of these cells in the lung tissue, masking or leveling their expansion in other, longer periods.

This limitation of the reproduced model did not allow us to evaluate the effectiveness of ruzam and the reference listed drug (sodium cromoglycate) by changes in the number of mast cells in the lung tissue and their DI *in vivo*. However, it did not reduce the informative value of assessing the anti-allergic effects of the compared pharmacological substances by other important criteria.

Ruzam increased the BALF volume returned, which indicated reduction of bronchial obstruction caused by allergic inflammation. Sodium cromoglycate was ineffective in this parameter.

A fundamentally different nature of the effect of ruzam and the reference listed drug on the cellular composition of BALF was revealed in the model of ovalbumin-induced airway inflammation. A combination of cytological changes caused by the use of ruzam can be assessed as suppression of eosinophilic and neutrophilic inflammation with simultaneous mobilization of effector cells in the immune response against pathogens (lymphocytes and macrophages). The reference listed drug, on the contrary, did not change the number of polymorphonuclear leukocytes in BALF, but caused a trend toward a decrease in the number of lymphocytes and macrophages.

At the same time, in the context of using both drugs, similar-amplitude suppression of the eosinophil and neutrophil infiltration of the interalveolar septa in the lung tissue samples was found.

The development of allergic inflammation in the airways was accompanied by hyperplasia of bronchus-associated lymphoid tissue consisting of many inducible lymphoid follicles. These ectopic lymphoid formations, currently classified as tertiary lymphoid tissues, are clusters of immune cells that resemble secondary lymphoid organs in their follicular structure. Inducible lymphoid follicles are formed in peripheral non-lymphoid tissues in response to the effects of various triggers, including antigens [22].

In the model of allergic inflammation reproduced by us, the development of tertiary lymphoid organs in the bronchi was caused by repeated ovalbumin inhalations to sensitized animals. The fact that a 6-day course of inhalations with ruzam or sodium cromoglycate at the final stage of allergic inflammation modeling did not change the density of bronchus-associated lymphoid follicles in the guinea pigs is quite understandable. It is known that even a single exposure of the airways to antigenic stimuli induces the development of bronchus-associated lymphoid structures that exist for at least 4 weeks after antigen clearance, reaching a peak in their development on days 8–12 [22]. Obviously, a course of inhalations with ruzam or the reference listed drug in our study was too short to cause noticeable regression of lymphoid follicles, which had formed earlier under the influence of allergenic stimuli and had not completed a natural course of their evolution and involution. Presumably, longer and / or earlier use of ruzam in such a model *in vivo* could confirm the anti-allergic effects of the drug by this parameter as well.

Cross-linking of high-affinity IgE receptors (FcRI) on mast cells by allergens causes rapid release of proinflammatory mediators that stimulate not only smooth muscle contraction and mucus secretion [23, 24], but also fibroblast proliferation and collagen synthesis, which leads to airway remodeling in asthma

and fibrosis of other tissues susceptible to allergic inflammation [25–27].

Mast cells along with basophils play an essential role in IgE-dependent allergic reactions [28]. When the latter are degranulated, a large number of phlogogenic mediators are released, provoking the development of asthma, allergic rhinitis, urticaria, and many other, not only allergic, diseases [30].

Given the above as well as the limitations that we encountered when assessing the number and functional state of mast cells in the *in vivo* model, the data obtained in the *in vitro* test systems on the pronounced ability of ruzam to suppress degranulation of mast cells and basophils induced by the histamine liberator and degranulation of basophils induced by ovalbumin in the animals sensitized with this antigen are very valuable. Therefore, the studied drug directly blocked both IgE-dependent and IgE-independent mechanisms of mast cell and basophil degranulation. It is worth noting that ruzam was effective in suppressing degranulation of cells in three mammalian species, one of which (rabbit) did not belong to rodents. This seems inspiring in terms of extrapolating the results obtained from animals to humans.

The experimental data described in this study are consistent with the results of clinical trials of ruzam in the treatment and prevention of allergic diseases highlighted in the recent review [4]. Biological effects of the drug in the *in vivo* and *in vitro* models confirm the ability of ruzam to suppress type 2 (Th2) immune responses underlying the pathogenesis of most allergic diseases.

Considering the chemical nature of ruzam (the complex of bacterial lipoproteins), we believe that the drug implements its biological / pharmacological effects through Toll-like receptors (TLR) of innate immunity. TLR2 and its heterodimers TLR1 / TLR2 and TLR / TLR6 are the most likely molecular targets of ruzam in this case. TLR2-mediated signals can both activate and regulate immune responses depending on the nature and dose of ligands (lipopeptides, lipoteichoic acids, proteoglycans), the variant of homo- or heterodimerization of this receptor, the initial state of the body, and a number of other factors [31].

In the context of interpretation of the obtained data, the previously revealed ability of lipopeptides to suppress allergic inflammation via the TLR2 signaling pathway in ovalbumin-induced asthma models *in vivo* is of interest. This pathway contributed to T2→T1 polarization of the predominant immune response and potentiation of immunoregulatory mechanisms [32, 33]. Defective TLR2, on the contrary, aggravated ultrastructural, cytological, and molecular signs of ovalbumin-induced type 2 inflammation in the airways of sensitized animals [34]. In *in vitro* test systems, TLR2 ligands suppressed IgE-dependent [35, 36] and IgE-independent mast cell degranulation [37]. We consider testing the hypothesis on the key role of TLR2 and its heterodimers in pharmacological effects of ruzam promising for further research not only to refine the molecular mechanisms of its effect, but also to expand and optimize the scope and methods of its clinical use.

CONCLUSION

The anti-allergic effect of ruzam, the extract from the culture of the thermophilic *S. aureus* strain C2, was confirmed in the test systems *in vivo* and *in vitro*. In particular, in the model of ovalbumin-induced allergic asthma in guinea pigs, the drug reduced the degree of airway obstruction and the severity of neutrophilic and eosinophilic inflammation, while mobilizing effector cells of the immune response against pathogens (lymphocytes and macrophages). And in the *in vitro* models, ruzam suppressed both IgE-independent degranulation of mast cells and basophils induced by the histamine liberator and IgE-dependent degranulation of basophils in the sensitized animals. A hypothesis was put forward about the TLR2-mediated nature of the main biological and pharmacological effects of the drug identified in this study and described in other scientific papers.

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Prognostic value of the inferior vena cava diameter, lung ultrasound, and the NT-proBNP level in patients with acute decompensated heart failure and obesity

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ABSTRACT

Aim. To evaluate the clinical and prognostic value of the inferior vena cava (IVC) diameter, the sum of B-lines according to lung ultrasound (LUS), and the NT-proBNP level in patients with acute decompensated heart failure (ADHF) and obesity.

Materials and methods. A single-center, prospective study included 162 patients with ADHF (66% men, age 68 ± 12 years, left ventricular ejection fraction (LVEF) 44 (35; 54)%, median level of NT-proBNP 4,246 (1,741; 6,837) pg / ml). 27.8% of patients were overweight, 55% of patients had obesity. Upon admission, all patients underwent a standard clinical and laboratory examination, including lung ultrasound with the calculation of the sum of B-lines, IVC ultrasound, and determination of the NT-proBNP level.

Results. Obese patients had a smaller sum of B-lines according to lung ultrasound than overweight patients and those with normal weight [33 (21–51); 38 (27–54), and 42 (30–58), respectively; $p = 0.002$] and a lower level of NT-proBNP [3,404 (1,630; 5,516); 4,458 (2,697; 5,969); 5,085 (2,871; 7,351) pg / ml, respectively, $p = 0.013$]. The IVC diameter did not differ significantly depending on body mass index (BMI): with obesity – 2.3 (1.9–2.8) cm, with overweightness – 2.3 (1.9–2.8) cm, and with normal weight – 2.2 (1.8–2.4) mm, $p = 0.324$.

According to the multivariate Cox regression analysis, the sum of B-lines > 7 at discharge (hazard ratio (HR) 8.90, 95% confidence interval (CI) 2.03–38.30, $p = 0.003$) and IVC > 2.4 cm at admission (HR 5.42, 95% CI 1.04–28.13, $p = 0.045$) were independently associated with a higher risk of 12-month mortality from cardiovascular disease.

Conclusion. Therefore, lung ultrasound with B-line quantification and assessment of the IVC diameter may be useful in obese patients with ADHF to stratify the risk of 12-month mortality from cardiovascular disease.

Keywords: ADHF, obesity, B-lines, inferior vena cava, NTproBNP

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Прогностическое значение диаметра нижней полой вены, ультразвукового исследования легких и NT-proBNP у пациентов с декомпенсацией хронической сердечной недостаточности и ожирением

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РЕЗЮМЕ

Цель: изучение клинического и прогностического значения диаметра нижней полой вены (НПВ), суммы В-линий по данным ультразвукового исследования (УЗИ) легких и NT-proBNP у пациентов с острой декомпенсацией хронической сердечной недостаточности (ОДХСН) и ожирением.

Материалы и методы. В одноцентровое проспективное исследование включены 162 пациента с ОДХСН (66% мужчин, возраст 68 ± 12 лет, фракция выброса левого желудочка (ФВ ЛЖ) 44 (35; 54)%, медиана уровня NT-proBNP 4 246 (1 741; 6 837) пг/мл). Имели избыточную массу тела 27,8% пациентов, 55% страдали ожирением. Всем пациентам при поступлении выполнены стандартное клинико-лабораторное обследование, включая УЗИ легких с подсчетом суммы В-линий, УЗИ НПВ, NT-proBNP.

Результаты. Пациенты с ожирением по сравнению с пациентами с избыточной и нормальной массой тела имели меньшую сумму В-линии при УЗИ легких [33 (21–51); 38 (27–54) и 42 (30–58) соответственно; $p = 0{,}002$], меньший уровень NT-proBNP [3 404 (1 630; 5 516); 4 458 (2 697; 5 969); 5 085 (2 871; 7 351) пг/мл соответственно, $p = 0{,}013$]. Диаметр НПВ существенно не отличался в зависимости от индекса массы тела: при ожирении – 2,3 (1,9–2,8) см, при избыточной массе тела – 2,3 (1,9–2,8) см, при нормальной массе тела – 2,2 (1,8–2,4) мм, $p = 0{,}324$.

По данным многофакторного регрессионного анализа Кокса, сумма В-линий более 7 при выписке (отношение рисков (ОР, hazard ratio (HR)) 8,90; 95%-й доверительный интервал (ДИ) 2,03–38,30; $p = 0{,}003$) и НПВ более 2,4 см при поступлении (ОР 5,42; 95%-й ДИ 1,04–28,13; $p = 0{,}045$) независимо ассоциировались с более высокой вероятностью 12-месячной смерти от сердечно-сосудистых заболеваний (ССЗ).

Заключение. Таким образом, УЗИ легких с подсчетом В-линий и оценка диаметра НПВ могут быть полезными у пациентов с ОДХСН и ожирением для стратификации по риску развития смерти от ССЗ в течение года.

Ключевые слова: ОДХСН, ожирение, В-линии, нижняя полая вена, NTproBNP

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом Медицинского института РУДН (протокол № 8 от 16.11.2021).

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INTRODUCTION

Congestion is one of the main causes of readmission and mortality in patients with chronic heart failure (CHF), regardless of left ventricular ejection fraction (LVEF). Recently, the role of lung ultrasound with an assessment of the sum of B-lines and the diameter of the inferior vena cava (IVC) in patients with acute decompensated heart failure (ADHF) for stratifying the risk of cardiovascular complications has been widely discussed [1]. The revealed relationship of body mass index (BMI) with both natriuretic peptides (NUP) and B-lines is of great clinical importance for obese patients [2].

Obese patients experience sodium retention, increased intravascular volume and blood pressure, and increased afterload, which can lead to adverse cardiac remodeling and contribute to development of heart failure (HF). In patients with dyspnea and / or edema and obesity, the diagnosis of HF is difficult, since the genesis of these symptoms is not always associated with the development of HF. In addition, there is evidence of an inverse correlation between BMI and the sum of B-lines on lung ultrasound in patients with acute or chronic HF [1, 2]. Therefore, search for effective methods for early diagnosis of congestion in patients with ADHF and obesity is relevant.

The aim of the study was to evaluate the clinical and prognostic value of the IVC diameter, the sum of B-lines according to lung ultrasound, and the NT-proBNP level in patients with ADHF and obesity.

MATERIALS AND METHODS

A single-center, prospective study included 162 patients with ADHF (66% men, average age 68 ± 12 years, median LVEF 44 (35; 54) %) (Table 1).

Table 1

Characteristics of patients with ADHF

Parameter	Value
Sex (male / female), *n* (%)	107 (66) / 55 (34)
Age, years, *M* ± *SD*	68 ± 12
Duration of HF, years, *Me* (*IQR*)	2 (0.3; 5)
NYHA functional class, *n* (%) II III IV	 5 (4) 79 (48) 78 (48)
Left ventricular ejection fraction (LVEF), %, *M* ± *SD*	40 ± 14
Hypertension, *n* (%)	157 (96.9)
Myocardial infarction in the medical history, *n* (%)	71 (44)
Atrial fibrillation, *n* (%)	97 (59.8)
Type 2 diabetes mellitus, *n* **(%)**	62 (38.2)
Chronic anemia, *n* (%)	36 (22.2)
NT-proBNP, pg / ml, *Me* (*IQR*)	4,246 (1,741; 6,837)
Creatinine (mg / dl)	107 (92; 136)
GFR CKD – EPI, ml/ min / 1.73m^2, *M* ± *SD*	54 (40; 69)
Glucose (mg / dl)	6.2 (5.3; 8)

Table 1 (continued)

Note: HF – heart failure, GFR CKD-EPI – glomerular filtration rate using the CKD – EPI equation.

The diagnosis of ADHF was established based on generally accepted criteria: a rapid increase in symptoms and / or signs of HF, structural and functional changes in the heart, and an increase in the NT-proBNP level. Exclusion criteria encompassed the presence of acute coronary syndrome, end-stage chronic kidney disease, cancer, severe anemia, fever, primary lung pathology (pneumonia, exacerbation of chronic obstructive pulmonary disease or bronchial asthma), and chest injury.

All patients underwent a routine clinical examination with an assessment of symptoms and /or signs of HF. The level of NT-proBNP was analyzed by the immunochemiluminescent assay (PathFast, Japan).

Echocardiography, lung ultrasound, and IVC diameter measurements were performed using the premium ultrasound machine Vivid E90 (GE Healthcare, USA). When performing standard echocardiography with an assessment of LVEF according to the Simpson's method, the maximum IVC diameter and collapsibility during the respiratory cycle were also measured from a subcostal view by a longitudinal scan (Fig. 1).

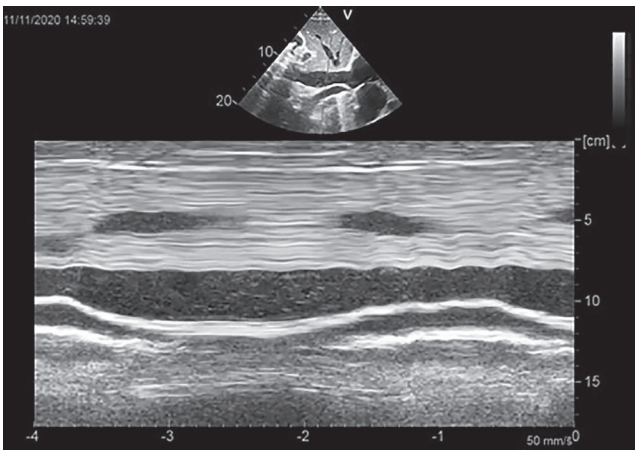

Fig. 1. Assessment of the IVC diameter

Lung ultrasound was performed in the first 48 hours from admission using an abdominal sensor in 8 zones along the anterolateral chest walls on both sides; the presence of B-lines in each zone was assessed, and their

sum was calculated (Fig. 2). The ultrasound pattern of the lungs was considered normal, corresponding to the absence of lung congestion, if the sum of B-lines was less than 5. If the sum of B-lines was more than 5, lung congestion was diagnosed [3].

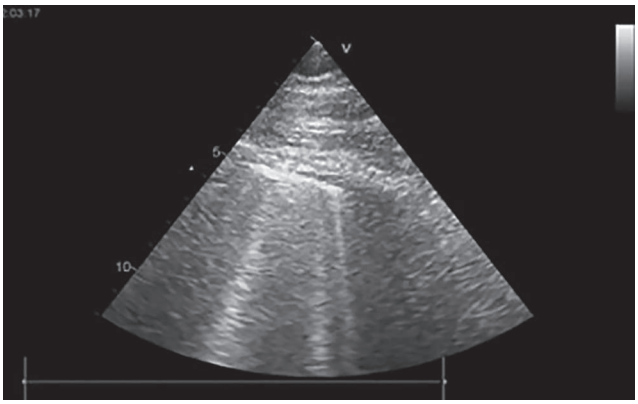

Fig. 2. Lung ultrasound. Multiple B-lines.

The follow-up period was 12 months. In telephone interviews 1, 3, 6, and 12 months after the discharge, outcomes were assessed (death from HF and readmission for decompensated HF). SPSS software (version 23.0) and MedCalc Version 19 were used for statistical data processing. Quantitative variables were described as the mean (M) and the standard deviation (*SD*) for normal distribution or as the median (*Me*) and the interquartile range (*IQR*) for non-normal distribution.

The Mann – Whitney test was used to assess significance of differences between the two groups of quantitative variables. Qualitative variables were represented by absolute (*n*) and relative (%) values. The Pearson's chi-squared test was used to compare the groups by the frequency of qualitative variables.

The receiver performance curve (ROC) was used to determine the predictive cut-off value for congestion parameters in normal-weight, overweight, and obese patients with HF, stratifying it into fatal and readmission values. The ratio of cases in the positive group (prevalence), sensitivity, and specificity were calculated. ROC curves were generated, and predictive cut-off values were defined as the point on the curve closest to 100% on the Y-axis. This was done by determining overload parameters associated with the highest sum of sensitivity and specificity [4].

The survival probability was estimated by constructing Kaplan – Meier survival curves; comparison was made using the log-rank test. The impact on the risk of mortality or readmission for HF was assessed by univariate and multivariate Cox regression analyses. The differences were considered significant at $p < 0.05$.

RESULTS

All patients were divided into 3 groups depending on BMI: patients with normal weight < 24.9 kg / m², overweight patients 25–29.9 kg / m², and obese patients ≥ 30 kg / m². Comparative characteristics of patients depending on BMI are presented in Table 2. Obese patients were younger, more likely to suffer from diabetes mellitus (DM), had arterial hypertension (AH), higher diastolic blood pressure (DBP), a smaller sum of B-lines, and a lower NT-proBNP level than overweight and normal-weight patients. There was no significant difference in the IVC diameter depending on BMI.

Table 2

Comparative characteristics of patients with ADHF depending on BMI at admission

Parameter	BMI, kg / m²			*p*
	< 24.9	25–29.9	≥ 30	
Sex (male / female)	14 (52)/13 (48)	35 (77.8)/10 (22.2)	58 (64.4)/ 32(35.5)	0.023
Age, years, *Me* (*IQR*)	71.8 (66–82)	71.3 (62–80)	65.9 (57–76)	0.005
	23.7 (23; 24)	27.8 (27; 29)	35 (32; 38)	<0.000
HF (NYHA), *n* (%): – II – III – IV	 19 (70) 8 (30)	 28 (62) 17 (38)	 6 (5.5) 31 (35.5) 53 (59)	0.007
Hypertension, *n* (%)	24 (88.8)	44 (97.8)	89 (98.8)	0.031
Diabetes mellitus, *n* (%)	6 (22.22)	14 (31.1)	42 (46.6)	0.009
Smoking, *n* (%)	9 (33.3)	15 (33.3)	36 (40)	0.404
Atrial fibrillation, *n* (%)	15 (55.5)	25 (55.6)	57 (63.3)	0.337
Heart rate, beats / min, *Me* (*IQR*)	87.7 (68–100)	87.8 (75–100)	91.9 (78–100)	0.126
SBP, *Me* (*IQR*)	135.1 (120–150)	139.6 (120–160)	144.8 (120–167)	0.093

Table 2 (continued)

Parameter	BMI, kg / m²			*p*
	< 24.9	25–29.9	≥ 30	
DBP, *Me* (*IQR*)	78.8 (70–84)	79.3 (40–80)	84.5 (80–90)	0.028
Dyspnea at rest, *n* (%)	10 (37)	16 (36)	28 (31)	0.490
Edema of the lower extremities at discharge, *n* (%)	2 (7.4)	13 (28.8)	38 (42.2)	0.031
LVEF, %	43.03 (28–55)	37.5 (23–47)	41.4 (31–50)	0.384
Sum of B-lines, *Me (IQR)*	42 (30; 58)	38 (27; 54)	33 (21; 51)	0.002
NT-proBNP, pg / ml, *Me* (*IQR*)	5,085 (2,871; 7,351)	4,458 (2,697; 11,969)	3,404 (1,630; 5,516)	0.013
Creatinine, mcmol / l, *Me* (*IQR*)	102 (89; 163)	107 (95; 160)	107 (91; 133)	0.596
GFR CKD – EPI, ml / min / 1,73 m²	53 (34; 67)	49 (39; 65)	56 (43; 70)	0.212
Glucose, mmol / l, *Me* (*IQR*)	5.7 (5.2; 7.2)	6.12 (4.9; 8.1)	6.4 (5.4; 8.4)	0.155
IVC diameter, cm, *M* ± *SD*	2.22 ± 0.51	2.33 ± 0.52	2.33 ± 0.52	0.324

Note: BMI – body mass index, HF – heart failure, SBP – systolic blood pressure, DBP – diastolic blood pressure, GFR CKD – EPI – glomerular filtration rate using the CKD – EPI equation.

During the 12-month follow-up, 85 events (52.4%) were identified, including 29 deaths (18%) and 56 repeated hospitalizations for CHF (34.4%). At the same time, in patients with obesity, the frequency of events was 47%: death – in 13% of cases and repeated hospitalization for CHF – in 34% of cases.

When constructing ROC curves to predict outcomes in obese patients, cut-off values were identified for the sum of B-lines according to lung ultrasound and IVC diameter (Table 3). There were no significant cut-off values for the NT-proBNP level in this group of patients. The univariate Cox regression analysis is presented in Table.4. The multivariate Cox regression analysis (which included sex, age, LVEF, HF functional class) confirmed independent predictive value for the risk of cardiovascular mortality during follow-up for the sum of B-lines >7 at discharge (hazard ratio (HR) 8.90, 95% confidence interval (CI) 2.03–38.30, $p = 0.003$) and IVC diameter > 2.4 cm at admission (HR 5.42, 95% CI 1.04–28.13, $p = 0.045$) in obese patients with ADHF.

Table 3

Cut-off values for predicting outcomes in obese patients

Parameter	Cut-off values	Sensitivity	Specificity	AUC	*p*
The sum of B-lines	>7	77	74	0.71	0.026
Diameter of IVC, cm	>2.4	78	68	0.73	0.0002
NT-proBNP, pg / ml	>5,053.8	70	77	0.70	0.059

Table 4

Univariate Cox regression analysis of the risk of death from cardiovascular disease in patients with heart failure, depending on the body mass index

Parameter	BMI < 24.9 kg / m², *n* = 6 (22%)		BMI = 25–29.9 kg / m², *n* = 11 (24%)		BMI ≥30 kg / m², *n* = 12 (44%)	
	OR	(95% CI) *p*	OR	(95% CI) *p*	OR	(95% CI) *p*
Atrial fibrillation	0.84	[0.17–4.20] 0.841	1.08	[0.51–2.28] 0.828	2.55	[1.13–5.73] 0.023
Inferior vena cava	0.87	[0.04–2.09] 0.930	1.04	[0.52–2.09] 0.894	2.92	[1.31–6.54] 0.009
LVEF < 40%	2.43	[0.44–13.3] 0.304	1.59	[0.93–2.68] 0.087	2.09	[1.05–4.16] 0.035
Sum of B-lines at discharge	1.09	[0.96–1.23] 0.162	1.03	[1.00–1.06] 0.024	1.06	[1.01–1.10] 0.011
Sum of B-lines > 7 at discharge	0.77	[0.12–3.69] 0.652	3.07	[1.44–6.53] 0.003	10.2	[2.76–37.7] <0.000
LogNTproBNP	1.25	[0.52–3.04] 0.609	1.59	[1.06–2.38] 0.023	1.82	[0.89–3.70] 0.098
NT-proBNP	1.00	[1.00–1.00] 0.779	1.00	[1.00–1.00] 0.033	1.00	[1.00–1.00] 0.064

Note: BMI – body mass index, OR – odds ratio.

The Kaplan – Meier curves for cumulative survival probability (death from cardiovascular disease) in obese patients depending on the sum of B-lines and IVC diameter are shown in Fig. 3, 4.

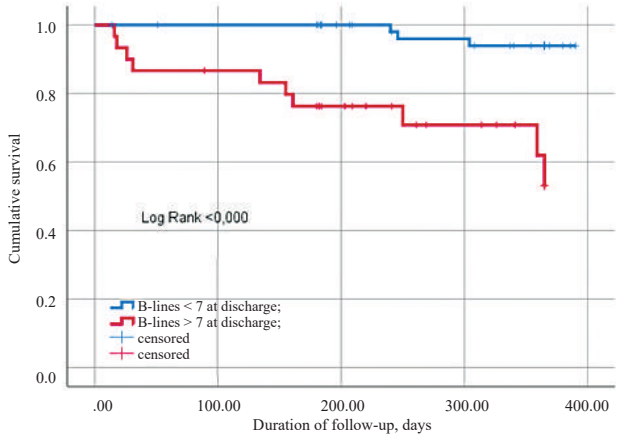

Fig. 3. Kaplan – Meier curves for cumulative survival probability (without death from cardiovascular disease) depending on the sum of B-lines > 7 in obese patients according to lung ultrasound at discharge

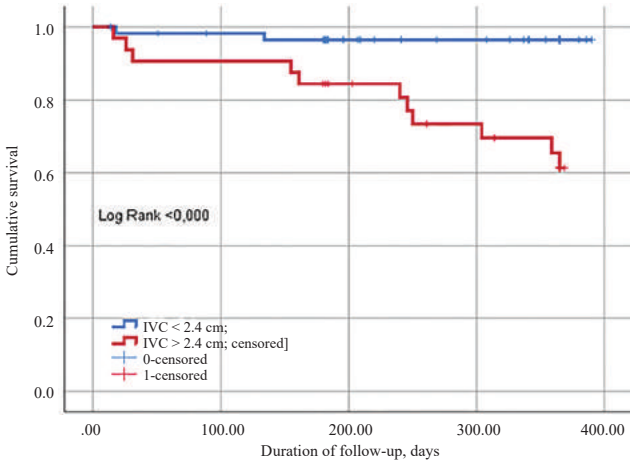

Fig. 4. Kaplan – Meier curves for cumulative survival probability (without death from cardiovascular disease) depending on the IVC diameter > 2.4 cm in obese patients according to lung ultrasound at discharge

DISCUSSION

In our study, in the patients with ADHF and obesity, an inverse relationship was found between BMI and both NTproBNP and the sum of B-lines. The IVC diameter did not differ significantly among patients depending on BMI. Our results are consistent with those of a recent study by A. Palazzuoli et al., who found an inverse correlation between BMI and the sum of B-lines according to lung ultrasound and no correlation between the IVC diameter and BMI for patients with acute or chronic HF [2, 5]. In addition, we noted lower levels of NTproBNP and fewer B-lines on lung ultrasound in obese patients, which was also demonstrated by E. Joyce et al. [6]. A smaller number of B-lines in obese patients may be associated with pronounced subcutaneous fat [1, 2, 7]. At the same time, to date, several hypotheses have been put forward to explain a smaller number of B-lines and lower levels of NTproBNP in patients with HF and obesity.

According to the literature, overweight is associated with a lower risk of death in patients with HF, which indicates a less severe course of the disease [1, 2, 8, 9]. On the other hand, patients with HF and obesity respond differently to HF treatment. Higher BMI can be associated with both fluid overload and excess fat accumulation. In the first case, the diuretic response can be much more effective, and in the second, on the contrary, it can even be reduced. A retrospective analysis of the DOSE study found that patients with a BMI > 30 kg / m² responded better to loop diuretics than non-obese patients [10]. It is also possible that the administration of intravenous loop diuretics rapidly clears lung congestion, but has less effect on the IVC diameter Since higher BMI is associated with a better prognosis, patients with obesity and HF may be less likely to develop lung congestion than individuals with lower BMI [11, 12]. Our study revealed the prognostic value of the sum of B-lines > 7 at discharge and the IVC diameter > 2.4 cm at admission for predicting 12-month mortality from cardiovascular disease in patients with HF and obesity, which is also consistent with the results of studies by other authors [1, 2, 13–15].

The limitations of the study are related to a relatively small number of patients. For lung ultrasound, we used an 8-zone protocol, which has good diagnostic accuracy and is more practical for clinical use in emergency situations. When using other protocols (4- or 28-zone protocols), other results are possible [16–18]. Long-term studies in a larger cohort of patients are required to further define the relationship between BMI, IVC diameter, and the sum of B-lines.

CONCLUSION

Lung ultrasound with B-line quantification and assessment of the IVC diameter may be useful in obese patients with ADHF to stratify the risk of 12-month mortality from cardiovascular disease.

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Prognostic value of myocardial flow reserve in patients with heart failure with preserved ejection fraction

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ABSTRACT

Aim. To study the prognostic value of myocardial blood flow (MBF) and myocardial flow reserve (MFR) parameters in patients with heart failure with preserved ejection fraction (HFpEF) and non-obstructive coronary artery disease (CAD) in risk stratification of HFpEF progression during a 12-month follow-up.

Materials and methods. The study included 58 patients with non-obstructive CAD and HFpEF (LVEF 62 [58; 66]%). Dynamic CZT-SRECT was used to evaluate MFR and MBF at rest (rest-MBF) and stress (stress-MBF). NT-proBNP levels were determined by the enzyme immunoassay. Diastolic dysfunction parameters were measured using 2D transthoracic echocardiography. Left ventricular systolic global longitudinal strain (GLS) was assessed using 2D speckle tracking.

Results. After a 12-month follow-up, the patients were retrospectively divided into 2 groups: group

1 (n = 11) included patients with an unfavorable course of HFpEF, group 2 (n = 47) encompassed patients with a favorable course of the disease. In group 1, the level of NT-proBNP was 3.8 times higher than in group 2 (284.5 [183.42; 716.73] and 1,071.4 [272.4; 2,168.1] pg / ml, respectively). MFR values in group 1 were lower by 45.4% ($p < 0.001$) than in group 2 (1.19 [0.86; 1.55] vs. 2.18 [1.7; 2.55], respectively). In group 1, rest-MBF levels were higher by 23.6% ($p = 0.046$) and stress-MBF was lower by 28.2% ($p = 0.046$) than in group 2. The multivariate regression analysis revealed that NT-proBNP levels (odds ratio (OR) 3.23; $p = 0.008$), GLS (OR 2.27; $p = 0.012$), and MFR (OR 8.09; $p < 0.001$) were independent predictors of adverse outcomes in HFpEF. Based on the ROC analysis, MFR levels ≤ 1.62 (AUC = 0.827; $p < 0.001$), GLS ≤–18 (AUC = 0.756; $p = 0.002$), and NT-proBNP ≥ 760.5 pg / ml (AUC = 0.708; $p = 0.040$) may be considered as markers of adverse outcomes. However, the combined determination of NT-proBNP and MFR had a greater significance (AUC 0.935; $p < 0.001$) in risk stratification compared with the monomarker model, while the addition of GLS did not increase the significance of the analysis.

Conclusion. Levels of NT-proBNP, GLS, and MFR may be used as non-invasive markers of an adverse course of HFpEF in patients with non-obstructive CAD, while the combined determination of NT-proBNP and MBF increases the prognostic value of the analysis.

Keywords: heart failure, preserved ejection fraction, myocardial flow reserve, prognosis, natriuretic peptide

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Cardiology Research Institute, Tomsk NRMC (Protocol No. 177 of 30.10.2018).

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Прогностическая роль резерва миокардиального кровотока у больных с сердечной недостаточностью с сохраненной фракции выброса

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РЕЗЮМЕ

Цель. Изучение роли параметров миокардиального кровотока (MBF) и резерва миокардиального кровотока (MFR) у пациентов с сердечной недостаточностью с сохраненной фракцией выброса (СНсФВ) и необструктивным поражением коронарных артерий (КА) в стратификации риска прогрессирования СНсФВ в течение 12 мес наблюдения.

Материалы и методы. В исследование включено 58 пациентов с необструктивным поражением КА и СНсФВ (ФВЛЖ 62 [58; 66]%). С помощью динамической CZT-SRECT оценивали показатели MFR, MBF в покое (rest-MBF) и при нагрузке (на фоне введения стресс-агента аденозинтрифосфата, stress-MBF). Уровни NT-proBNP определяли с помощью иммуноферментного анализа. Параметры диастолической дисфункции измеряли с помощью двумерной трансторакальной эхокардиографии. Систолическая глобальная продольная деформация ЛЖ (GLS) оценивалась с помощью 2D-speckle tracking.

Результаты. Через 12 мес наблюдения больные ретроспективно были разделены на две группы: в группу 1 (n = 11) вошли больные с неблагоприятным течением СНсФВ, в группу 2 (n = 47) – с благоприятным. В группе 1 уровень NT-proBNP был выше в 3,8 раза, чем в группе 2 (284,5 [183,42; 716,73] и 1071,4 [272,4; 2168,1] пг/мл соответственно). Значения MFR были ниже в группе 1 на 45,4% (p < 0,001), чем в группе 2 (1,19 [0,86; 1,55] vs 2,18 [1,7; 2,55] соответственно). Уровни rest-MBF были выше на 23,6% (p = 0,046), а stress-MBF ниже на 28,2% (p = 0,046) в группе 1, чем в группе 2. При проведении многофакторного регрессионного анализа уровни NT-proBNP (отношение шансов (ОШ) 3,23; p = 0,008), GLS (ОШ 2,27; p = 0,012) и MFR (ОШ 8,09; p < 0,001) оказались независимыми предикторами неблагоприятного течения СНсФВ. По данным ROC-анализа, уровни MFR ≤1,62 (AUC = 0,827; p < 0,001), GLS ≤–18 (AUC = 0,756; p = 0,002) и NT-proBNP ≥760,5 пг/мл (AUC = 0,708; p = 0,040) можно рассматривать в качестве маркеров неблагоприятных исходов. Однако комбинированное определение NT-proBNP с MFR обладало большей значимостью (AUC 0,935; p < 0,001) в стратификации риска по сравнению с мономаркерной моделью, тогда как добавление GLS не увеличивало значимость анализа.

Заключение. Уровни NT-proBNP, GLS и MFR могут использоваться в качестве неинвазивных маркеров неблагоприятного течения СНсФВ у пациентов с необструктивным поражением КА, при этом комбинированное определение NT-proBNP и MBF увеличивает прогностическую значимость анализа.

Ключевые слова: сердечная недостаточность, сохраненная фракция выброса, резерв миокардиального кровотока, прогноз, натрийуретический пептид

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Heart failure (HF), which was designated as a new epidemic in 1997, remains a serious and dire clinical and public health problem worldwide [1]. Approximately 50% of patients with HF are diagnosed with preserved left ventricular ejection fraction (LVEF) [2], and the prevalence of HF with preserved ejection fraction (HFpEF) increases by about 1% annually compared to HF with reduced LVEF [3]. At the same time, 5-year survival of patients with HFpEF is comparable to some types of non-hematological cancers [4], and the cost of treatment is associated with high economic costs, amounting to 1–2% of the total healthcare expenditures. According to forecasts, by 2030, the annual cost of treatment in this cohort of patients will reach 69.8 billion dollars [5].

Currently, the mechanisms of development and progression of HFpEF remain not fully understood [6]. At the same time, a lack of an accurate understanding of its pathophysiology determines a lack of adequate therapy according to current guidelines [7]. Recently, a new innovative theory of the development and progression of HFpEF has been proposed, which is based on coronary microvascular dysfunction (CMD) [8]. The results of a number of international studies using invasive or non-invasive diagnostic methods support the assumption that CMD occurs much more often than previously established, including patients with HFpEF [9]. V.L. Murthy et al. found that 53% of patients with non-obstructive coronary artery disease (CAD) and angina showed signs of mental stress-induced myocardial ischemia [10]. According to a meta-analysis of 56 studies involving 14,427 patients, the proportion of patients with CMD in the general population was 41% [9], while the prevalence of CMD in patients with HFpEF increased to 75–85% [11, 12].

Myocardial flow reserve (MFR), quantified as the ratio of hyperemic myocardial blood flow to resting blood flow, is used for a functional assessment of ischemia in large and small vessels. In the absence of subepicardial coronary artery occlusion, it is a marker of CMD [13]. Currently, magnetic resonance imaging (MRI) of the heart and positron emission tomography (PET) are among the main methods for diagnosing CMD, but their use for assessing myocardial perfusion parameters has not found wide application in clinical practice due to complexity and high cost of the methods [14, 15]. Another method for determining absolute perfusion parameters is dynamic myocardial perfusion single-photon emission computed tomography (SPECT) [16]. This technique has appeared relatively recently, with the advent of a new class of gamma cameras equipped with cadmium – zinc – telluride (CZT) detectors. The method, along with PET, has been sufficiently tested and validated, and is also more accessible for visualizing microcirculatory changes in the coronary bed [17]. However, the predictive value of MFR and MBF parameters, obtained using CZT detectors, in risk stratification of HFpEF progression has not yet been evaluated.

The aim of the study was to investigate the prognostic value of MBF and MFR parameters in patients with HFpEF and non-obstructive CAD in risk stratification of HFpEF progression during a 12-month follow-up.

MATERIALS AND METHODS

The study was performed in accordance with the Declaration of Helsinki and approved by the local Ethics Committee at the Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences (Protocol No. 177 of 30.10.2018). An informed written consent was

obtained from all patients prior to enrollment in the study.

Inclusion criteria: 1) non-obstructive (< 50%) CAD; 2) LVEF ≥ 50% measured by echocardiography; 3) left ventricular diastolic dysfunction (DD) /increased left ventricular filling pressure according to echocardiography; 4) sinus rhythm; 5) NT-proBNP ≥ 125 pg / ml; 6) a signed informed consent to participate in the study.

Exclusion criteria: 1) previous myocardial infarction; 2) elective coronary revascularization and / or previous coronary revascularization; 3) systolic blood pressure > 160 mm Hg; 4) symptomatic hypotension with the mean systolic blood pressure < 90 mm Hg; 5) second- or third-degree atrioventricular block, sick sinus syndrome; 6) persistent or chronic atrial fibrillation and / or atrial flutter; 7) valvular insufficiency or aortic stenosis ≥ grade 2; 8) hypertrophic and dilated cardiomyopathy; 9) previous pulmonary embolism with increased pulmonary hypertension ≥ 45 mm Hg; 10) severe form of bronchial asthma and / or chronic obstructive pulmonary disease; 11) pathology of the thyroid gland; 12) glomerular filtration rate (CKD-EPI) < 30 ml / min / m².; 13) Child-Pugh class C liver failure; 14) acute and chronic inflammatory diseases of the heart; 15) hemoglobin level < 100 g / dl; 16) stroke or transient ischemic attack within 90 days prior to inclusion in the study; 17) obesity (body mass index > 35 kg / m²); 18) life-threatening uncontrolled arrhythmias.

Preparation for coronary computed tomography angiography (CCTA) was carried out according to a standard protocol. The preparation included taking beta blockers and prednisolone, avoiding caffeine-containing drinks and foods, excluding glucophage (metformin), viagra, etc., painkillers (advil or motrin). In addition, patients were instructed about the contraindications of the procedure associated with allergic reactions, pregnancy and kidney disease. Before each scan, heart rate and blood pressure were assessed. All patients received 0.5 mg nitroglycerin sublingual.

For contrast-enhanced scanning, 70–90 ml of a non-ionic contrast agent (iopamidol 370 mg, Bracco Diagnostics, Italy) was injected intravenously through an 18G catheter inserted through the cubital vein at the rate of 5–5.5 ml / s followed by 60 ml of 0.9% NaCl. Axial images, curvilinear multiplanar and transverse reformations, and thin-slab maximum intensity projections were used to analyze the data set. All studies were analyzed on the hybrid tomograph (Advantage Workstation 4.6, GE Healthcare). According to the modified classification proposed by the American Heart Association, coronary arteries were divided into 16 segments [18].

Preparation of patients for dynamic myocardial perfusion SPECT, study protocol, and recording and processing of static and dynamic scintigraphic data are described in previous works [16]. Twenty-four hours prior to the study, beta blockers, nitrates, calcium channel antagonists, caffeine, and methylxanthine derivatives were discontinued. Studies were performed in the morning, on an empty stomach, against the background of sinus rhythm, according to a two-day rest – stress protocol using the radiopharmaceutical 99mTc-methoxy-isobutyl-isonitrile (99mTc-MIBI), which was administered intravenously as a bolus at a dose of 260–444 MBq. To perform the study under stress, the stress agent adenosine triphosphate (ATP) was used, which was administered using an intravenous infusion pump at a dose of 160 μg / kg / min for 4 minutes.

To correct the attenuation, low-dose computed tomography of the chest was performed. All studies were performed on the Discovery NM/CT 570c hybrid tomograph (GE Healthcare, Milwaukee, WI, USA) equipped with a gamma camera with highly sensitive CZT detectors. The total effective radiation dose in the study (rest and pharmacological stress test) was ~6.25 mSv.

The resulting scintigraphic images were processed on the specialized Xeleris II workstation (GE Healthcare, Haifa, Israel). Myocardial perfusion, MBF, and MFR were assessed using specialized software Corridor 4DM SPECT and 4DM Reserve v.2015 (INVIA, Ann Arbor, MI, USA). For processing quantitative characteristics, the Net Retention model was used with attenuation correction.

According to myocardial perfusion SPECT data, standard semi-quantitative indices of myocardial perfusion impairment were determined: Summed Stress Score (SSS) – the sum of points during exercise, Summed Rest Score (SRS) – the sum of points at rest, Summed Difference Score (SDS) – the difference between exercise and rest, and also quantitative parameters: Stress Myocardial Blood Flow (stress-MBF) – myocardial blood flow during exercise, Rest Myocardial Blood Flow (rest-MBF) – myocardial blood flow at rest, and Myocardial Flow Reserve (MFR).

Philips Affiniti 70 ultrasound system was used to perform 2D transthoracic echocardiography.

All examinations were performed by one highly qualified specialist. The assessment of left ventricular diastolic dysfunction (LVDD) was based on 4 main parameters: early diastolic velocity of the lateral wall of the left ventricle (lateral e'), the average ratio of early diastolic mitral valve flow velocity to the average early diastolic mitral annular velocity (E/e'), left atrial volume index, and peak tricuspid regurgitation velocity [19]. LVDD was diagnosed in the presence of ≥ 3 abnormal values. LV systolic global longitudinal strain (GLS) was assessed using 2D speckle tracking.

Blood samples were obtained by venipuncture; adequate samples were centrifuged. Serum was separated and stored at –24 °C with one freeze – thaw cycle. The level of NT-proBNP was determined by the enzyme-linked immunosorbent assay (ELISA) using the Biomedica ELISA kit (Austria). Adverse outcomes were defined as the time to new symptoms / signs or aggravating symptoms/signs of HF, hospitalization for decompensated HFpEF, or death.

Statistical processing of the study results was performed using STATISTICA 10.0 and MedCalc 11.5.0.0 software. The data were presented as the median and the interquartile range *Me* (Q_{25}–Q_{75}). To test statistical hypotheses in the analysis of quantitative variables, the Mann – Whitney test was used to compare two independent samples. When analyzing qualitative variables, contingency tables were analyzed using the Pearson's $\chi 2$ test. If there were cells with an expected frequency of less than 5, a two-tailed Fisher's exact test or Yates' correction (for 2x2 tables) was applied. To identify predictors of an unfavorable course of HFpEF, a ROC analysis was used with construction of characteristic curves and calculation of area under the curve (AUC). To identify factors that have a significant impact on the course and prognosis of the disease, a multivariate analysis was performed with calculation of an odds ratio (OR) with a 95% confidence interval (CI). The critical significance level *p* for all statistical procedures was taken equal to 0.05.

RESULTS

After 12 months of follow-up, the patients were retrospectively divided into 2 groups: group 1 (n = 11) included patients with an unfavorable course of HFpEF, group 2 (n = 47) encompassed patients with a favorable course of the disease (Table 1). Adverse cardiovascular events recorded during the follow-up period are shown in Fig. 1.

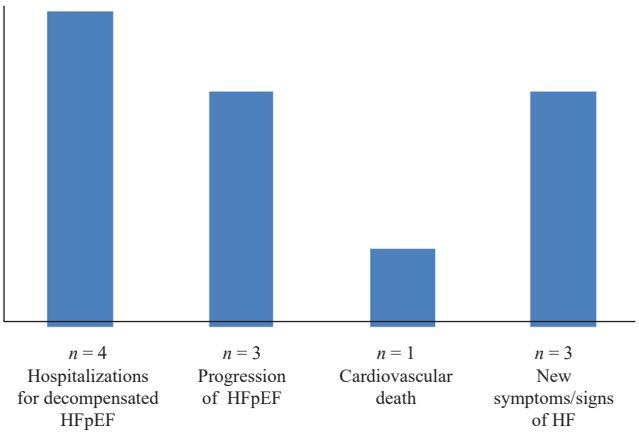

Fig. 1. Frequency of adverse cardiovascular events during 12-month follow-up

According to the main clinical and demographic characteristics, the groups were comparable, except for the NT-proBNP values ($p < 0.001$). In group 1, the level of NT-proBNP was 3.8 times higher than in group 2 (284.5 [183.42; 716.73] and 1,071.4 [272.4; 2,168.1] pg / ml, respectively).

In patients with an unfavorable course of HFpEF, the absolute value of GLS was lower by 27.1% (p = 0.003) than in individuals with a favorable course of the disease (–14.5 [12; 18.9]% and –19.9 [14; 21.4] %, respectively; p = 0.003). Septal e′ was lower by 23.6% (p = 0.008) in group 1 than in group 2 (5.5 [4.9; 6.7) versus 7.2 (6.9; 8.01) cm, respectively). E/e' values were higher by 14.7% (p = 0.041) and LAVI was higher by 17.8 (p = 0.021) in patients with adverse outcomes of HFpEF compared to patients with a favorable course of the disease (Table 2).

Semi-quantitative parameters of LV myocardial perfusion did not differ significantly in the studied groups. MFR values were lower in group 1 by 45.4% ($p < 0.001$) than in group 2 (1.19 [0.86; 1.55] vs. 2.18 [1.7; 2.55], respectively). The value of rest-MBF in patients with an unfavorable course of HFpEF was higher by 30.1% than that in the group with a favorable course of the disease (p = 0.046), while stress-MBF in group 1 was lower by 28.2 % (p = 0.014) than in group 2 (Table 3).

MFR and rest-MBF levels were correlated with NT-proBNP levels (r = –0.368; p = 0.007 and r = 0.354; p = 0.042, respectively). MFR values were also correlated with LAVI (r = –0.464; p = 0.001), lateral e' (r = 0.314, p = 0.012), and GLS (r = 0.504, p = 0.009), while rest-MBF was correlated with E/e' (r = 0.512; p = 0.002).

Table 1

Clinical and demographic characteristics of patients depending on the course of HFpEF

Parameter	Group 1, *n* = 11	Group 2, *n* = 48	*p*
Age, years, *Me* (Q_{25}–Q_{75})	62 (54.0; 67.0)	60.0 (53.0; 68.0)	0.124
Men, *n* (%)	7 (63.6)	29 (60.4)	0.912
Body mass index, kg / m^2, *Me* (Q_{25}–Q_{75})	32.4 (29.9; 34.8)	30.19 (27.8; 33.3)	0.174
Hypertension, *n* (%)	8 (72.7)	32 (66.7)	0.257
Diabetes mellitus, *n* (%)	3 (27.3)	14 (29.2)	0.863
COPD, *n* (%)	2 (18.2)	11 (22.9)	0.315
Smoking, *n* (%)	3 (27.3)	10 (20.8)	0.311
GFR, ml / min / 1.73 m^2, *Me* (Q_{25}–Q_{75})	69.8 (57.0; 78.5)	71.0 (59.0; 81.0)	0.745
Total cholesterol, mmol / l, *Me* (Q_{25}–Q_{75})	4.34 (3.76; 5.23)	4.67 (3.98; 5.54)	0.976
HbA1c, %, *Me* (Q_{25}–Q_{75})	5.7 (5.2; 6.8)	5.4 (5.3; 6.9)	0.721
LDL-C, mmol / l, *Me* (Q_{25}–Q_{75})	3.19 (1.78; 3.65)	1.65 (1.99; 3.34)	0.457
HDL-C, mmol / l, *Me* (Q_{25}–Q_{75})	1.07 (0.85; 1.31)	1.06 (0.96; 1.26)	0.896
Triglycerides, mmol / l, *Me* (Q_{25}–Q_{75})	1.69 (1.23; 1.97)	1.67 (1.22; 1.92)	0.235
Hemoglobin, g / dl, *Me* (Q_{25}–Q_{75})	133 (127; 143)	135 (128; 142)	0.675
Potassium, mmol / l, *Me* (Q_{25}–Q_{75})	4.56 (4.01; 5.12)	4.87 (4.43; 5.21)	0.346
Fibrinogen, g / l, *Me* (Q_{25}–Q_{75})	3.27 (3.14; 3.14)	3.17 (2.86; 3.43)	0.844
NT-proBNP, pg / ml, *Me* (Q_{25}–Q_{75})	1,701.4 (272.4; 2,168.1)	284.5 (183.4; 716.7)	<0.001

Note: HbA1c – glycated hemoglobin; GFR – glomerular filtration rate according to the CKD-EPI equation; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; COPD – chronic obstructive pulmonary disease

Table 2

Echocardiography parameters depending on the course of HFpEF, *Me* (Q_{25}–Q_{75})

Parameter	Group 1, *n* = 11	Group 2, *n* = 48	*p*
Left ventricular ejection fraction, %	59.5 (56; 62.5)	61 (59; 64)	0.456
End-systolic dimension, mm	43 (38; 47)	41.5 (36.5; 45.5)	0.544
End-diastolic dimension, mm	56.0 (49.5; 59.0)	54.5 (47.5; 57.5)	0.398
LVMMI, g / m^2	99.0 (88.5; 112.5)	97 (85.5; 109.5)	0.276
E/A ratio	1.04 (0.79; 1.34)	0.99 (0.74; 1.2)	0.516
Lateral e′, cm / m	5.5 (4.9; 6.7)	7.2 (6.9; 8.01)	0.008
Peak TRV, m / s	2.99 (2.95; 3.21)	2.92 (2.8; 3.11)	0.056
E/e’	14.5 (13.5; 15.0)	13 (12; 14)	0.041
LAVI, ml / m^2	38.3 (35.7; 51.1)	31.48 (29.5; 47.9)	0.021
LV global longitudinal strain, %	–14.5 (–12; –18.9)	–19.9 (14; 21.4)	0.003

Note: E/A – the ratio of the maximum blood flow velocity in the rapid ventricular filling phase to the maximum flow velocity in atrial systole; E/e’ – the ratio of the early mitral inflow velocity to tissue Doppler e′; LVMMI – left ventricular myocardial mass index; LAVI – left atrial volume index; TRV – tricuspid regurgitation velocity; lateral e’ – early diastolic velocity of the lateral wall of the left ventricle.

Table 3

Coronary flow reserve and myocardial blood flow parameters depending on the course of HFpEF, *Me* (Q_{25}–Q_{75})

Parameter	Group 1, *n* = 11	Group 2, *n* = 48	*p*
Stress-MBF, ml / min / g	1.07 (0.57; 1.22)	1.49 (1.09; 1.71)	0.014
Rest-MBF, ml / min / g	0.72 (0.52; 1.22)	0.55 (0.47; 0.77)	0.046
MFR	1.19 (0.86; 1.55)	2.18 (1.7; 2.55)	<0.001
SSS	3 (0; 4)	2 (0; 4)	0.563
SRS	0 (0; 1)	0 (0; 2)	0.423
SDS	2 (2; 3)	1 (0; 3)	0.221

Note: MFR – myocardial flow reserve; standard semi-quantitative indices of impaired myocardial perfusion: SSS – summed stress score; SRS – summed rest score; SDS – summed difference score.

In the multivariate regression analysis, NT-proBNP levels (OR 3.23; 95% CI 1.76–6.78; p = 0.008), GLS (OR 2.27; 95% CI 1.15–4.65; p = 0.012, and MFR (OR 8.09; 95% CI 5.12–19.98; p < 0.001) were independent predictors of an unfavorable course of HFpEF. According to the ROC analysis, MFR levels ≤ 1.62 (AUC = 0.827; p < 0.001), GLS ≤ –18 (AUC = 0.756; p = 0.002), and NT-proBNP ≥ 760.5 pg / ml (AUC = 0.708; p = 0.040) may be considered as markers of poor outcomes (Fig. 2, *a*). Comparison of the ROC curves showed no difference in the predictive value of the markers (p = 0.953) (Fig. 2, *b*). However, the combined determination of NT-proBNP with MFR was more significant (AUC = 0.935; p < 0.001) in risk stratification compared to the monomarker model (Fig. 3), whereas the addition of GLS (AUC = 0.885; p = 0.570) did not significantly increase the predictive value of the analysis.

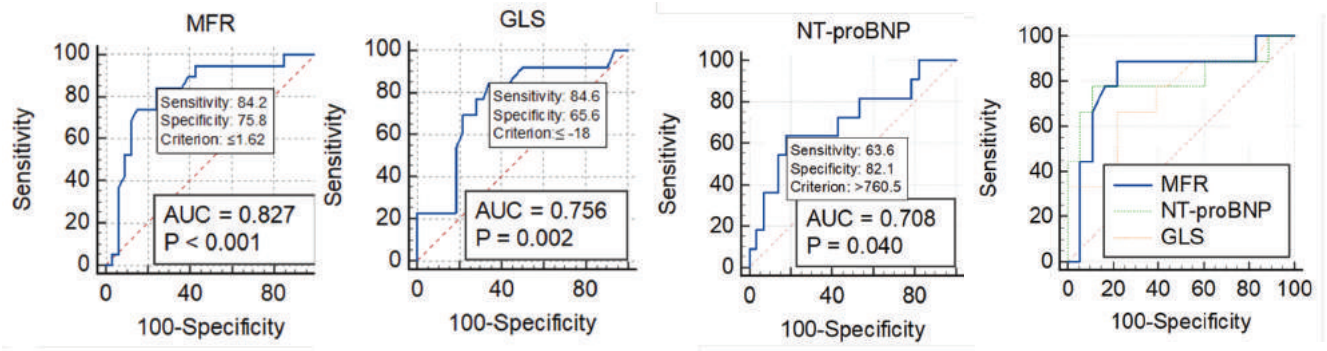

Fig. 2. Sensitivity and specificity of MFR, NT-proBNP, and GLS values in risk stratification of the unfavorable course of HFpEF in patients with non-obstructive coronary artery disease (ROC analysis)

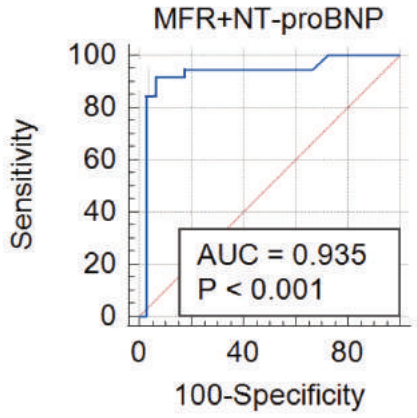

Fig. 3. Combined determination of MFR and NT-proBNP in the risk stratification of the unfavorable course of HFpEF in patients with non-obstructive coronary artery disease (ROC analysis)

DISCUSSION

The results of recent studies have shown that CMD may play an important role in the pathogenesis of HFpEF [12, 20], possibly because impaired perfusion causes damage to cardiomyocytes, which leads to a decrease in the functional reserve of the heart [21–24] and development of myocardial fibrosis [16]. Despite strong evidence in support of CMD, only a few studies have evaluated its role as a predictor of adverse HFpEF outcomes [15–19, 25, 26], and only one study found that a decrease in MFR on dynamic SPECT was an independent predictor of a high risk of major adverse cardiac events (MACE) [27].

The results of the COURAGE and ISCHEMIA studies showed that coronary artery revascularization was not associated with significant reduction of the incidence of MACE [28, 29]. The first international COVADIS study provided new evidence that the presence of CMD is an important problem and portends a high risk of MACE [30]. It was later found that impaired MFR is associated with an increased risk of all-cause mortality and the development of MACE [31]. J. Schroder et al. showed that CMD, assessed by Doppler echocardiography as coronary blood flow velocity in the anterior descending coronary artery, was also associated with an increased risk of rehospitalization for angina and all-cause mortality [32]. S. Kato et al. obtained and analyzed MRI-assessed MFR data in 163 patients with HFpEF (73 ± 9 years; 86 [53%] women). MFR values were significantly lower in patients with HFpEF and adverse cardiovascular events than in patients without them (1.93 ± 0.38 vs. 2.67 ± 0.52, p < 0.001) and were a predictor of cardiovascular death and hospitalizations for HF [33]. At the same time, significant negative correlations were found between MFR and global circumferential strain (r = –0.29, p< 0.001), GLS

(r = –0.33, p < 0.001), right ventricular longitudinal strain (r = –0.26, p < 0.001), and serum BNP levels (r = -0.32, p < 0.001) [34].

A. Ahmed et al. showed that the severity of microcirculatory disorders is inversely proportional to the LV filling pressure, especially during exercise [22]. In another study including patients with suspected CAD with preserved LVEF who underwent PET, a decrease in MFR was associated with the presence of DD (OR 2.58; 95% CI 1.22–5.48) and a high risk of hospitalization due to decompensated HFpEF (OR 2.47; 95% CI 1.09–5.48) [17]. Patients with reduced MFR levels on PET and DD demonstrated a more than five-fold increased risk of hospitalization due to decompensated HFpEF (p < 0.001). However, J.H. Lam et al. found no relationship between echocardiography parameters of LVDD and CMD, assessed using myocardial contrast echocardiography at rest [33]. In another study that included patients with systolic dysfunction (LVEF < 35%) and non-obstructive CAD, MFR parameters were associated with E/e' values [34].

We have demonstrated for the first time that patients with an unfavorable course of HFpEF had lower MFR values according to dynamic SPECT, probably due to more pronounced changes in the microvascular bed. MFR and rest-MBF levels were correlated with NT-proBNP levels (r = –0.368; p = 0.007 and r = 0.354; p = 0.042, respectively). MFR values were also correlated with LAVI (r = –0.464; p = 0.001), lateral e' (r = 0.314, p = 0.012), and GLS (r = 0.504, p = 0.009), and rest-MBF was correlated with E/e' (r = 0.512; p = 0.002).

This suggests that factors that tip the balance towards cardiomyocyte damage in patients with existing CMD may worsen myocardial mechanics and increase the risk of HFpEF progression even in non-obstructive CAD. In particular, CMD leads to a decrease in the bioavailability of nitric oxide, and an increase in profibrotic cytokine signaling may contribute to a decrease in coronary microvascular rarefaction and an increase in myocardial fibrosis observed in HFpEF [17]. This interaction of disorders can synergize vascular and ventricular rigidity in CMD [35].

On the one hand, in patients with CMD, diffuse myocardial fibrosis leads to an endothelium-dependent increase in peripheral vascular resistance and an increase in resting blood flow. On the other hand, CMD associated with chronic systemic inflammation may contribute to periarteriolar fibrosis and microvascular rarefaction, leading to a decrease in stress-MBF [36]. The correlation of dynamic SPECT parameters with the biomarker of volume overload (NT-proBNP) and diastolic function parameters indicates a closer relationship between these processes, which, in particular, indicates the pathogenesis of HFpEF [8]. In addition, we found that the levels of NT-proBNP (OR 3.23; p = 0.008), GLS (OR 2.27; p = 0.012), and MFR (OR 8.09; p < 0.001) were independent predictors of an unfavorable course of HFpEF. According to the ROC analysis, MFR levels ≤ 1.62 (AUC = 0.827; p < 0.001), GLS ≤ –18 (AUC = 0.756; p = 0.002). and NT-proBNP ≥ 760.5 pg / ml (AUC = 0.708; p = 0.040) can be considered as markers of adverse outcomes. However, the combined determination of NT-proBNP with MFR was more significant (AUC = 0.935; p < 0.001) in risk stratification compared to the monomarker model, while the addition of GLS (AUC = 0.885; p = 0.570) did not significantly increase the predictive value of the analysis.

CONCLUSION

The levels of NT-proBNP, GLS, and MFR can be used as non-invasive markers of an unfavorable course of HFpEF in patients with non-obstructive CAD, while the combined determination of NT-proBNP and MBF has a greater prognostic value in risk stratification of an unfavorable course of this pathology during 12-month follow-up.

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Respiration in isolated cardiomyocytes and microviscosity of their membranes in rats of different ages with heart failure

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ABSTRACT

Background. According to the latest epidemiological data, heart failure (HF) is diagnosed in 10% of adult population over 70 years old. However, currently this diagnosis is being increasingly made in young and middle-aged people. The pathogenesis of HF may be based on a decrease in respiration in cardiomyocytes with age, which affects the function of energy-dependent processes in cells.

Aim. To study respiration in cardiomyocytes and microviscosity of their membranes in rats aged 2 and 15 months with heart failure.

Materials and methods. The study was carried out on male Wistar rats. The animals were divided into 4 groups: 2 groups of intact rats aged 2 and 15 months (n = 12) and 2 groups of animals of similar ages (n = 10) with a model of HF. In the latter, HF formed by day 28 after a double subcutaneous injection of isoproterenol hydrochloride at a dose of 170 mg / kg with an interval of 24 hours. Isolated cardiomyocytes were obtained from enzyme-washed rat hearts. Cell respiration was studied in a thermostated chamber in an incubation medium supplemented with phosphorylation (ADP) and oxidation (succinate) substrates. The respiratory control (RC) ratio was calculated by dividing V3 respiration state to V4. Membrane microviscosity characteristics were assessed by the eximerization coefficient of pyrene fluorescence in the areas of protein – lipid and lipid – lipid interactions.

Results. RC in cardiomyocyte membranes of intact animals did not change with age. In 2-month-old rats with HF, respiratory control ratio (RCR) did not change compared with the intact age-matched controls. In 15-month-old rats with HF, there was a significant decrease in RC of cardiomyocytes (CM) compared with the intact animals of this age and 2-month-old rats with HF. An age-dependent decrease in the microviscosity of CM membranes in the areas of lipid – lipid interactions and no significant changes in the parameter at the sites of protein – lipid interactions were noted. In 2-month-old animals with HF, the microviscosity of CM membranes in the areas of protein – lipid interactions significantly decreased, and in 15-month-old rats it increased, compared with the intact controls. When carrying out an intergroup comparison, an age-dependent increase in the microviscosity of CM membranes in the areas of protein – lipid interactions and no differences in the parameter in the areas of lipid – lipid interactions were revealed.

Conclusion. In the intact rats, the absence of significant changes in respiration with age was revealed. In the 15-month-old animals with HF, respiration in CM was significantly lower than in the intact controls and 2-month-old animals with HF. These changes may be due to the differences in the membrane microviscosity characteristics in different periods of ontogenesis.

Keywords: cardiomyocyte respiration, membrane microviscosity, age-dependent changes, rats

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Активность дыхания изолированных кардиомиоцитов и микровязкость их мембран у крыс разных возрастов при сердечной недостаточности

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РЕЗЮМЕ

Актуальность. По последним эпидемиологическим данным, сердечная недостаточность (СН) диагностирована у 10% взрослого населения старше 70 лет, при этом все чаще данный диагноз ставят лицам молодого и среднего возраста. В основе патогенеза СН может лежать снижение дыхания в кардиомиоцитах с возрастом, что отражается на работе энергозависимых процессов в клетках.

Цель. Изучить дыхательную активность и микровязкость мембран кардиомиоцитов (КМ) крыс в возрасте 2 и 15 мес при сердечной недостаточности.

Материалы и методы. Исследование проведено на самцах крыс линии Wistar. Сформировано четыре группы животных: две группы интактных крыс в возрасте 2 и 15 мес (n = 12) и две группы животных аналогичных возрастов (n = 10), у которых моделировали развитие СН, формировавшуюся к 28-м сут после двукратного подкожного введения изадрина гидрохлорида в дозе 170 мг/кг с интервалом 24 ч. Изолированные кардиомиоциты получали из промытого ферментами сердца. Изучение активности дыхания клеток проводили в термостатируемой камере в инкубационной среде с добавлением субстратов фосфорилирования (АДФ) и окисления (сукцинат). Рассчитывали коэффициент дыхательного контроля (ДК) как отношение скоростей убыли кислорода в метаболических состояниях V3 и V4. Микровязкостные характеристики мембран оценивали по коэффициенту эксимеризации флуоресцентного зонда пирен в зонах белок-липидных и липид-липидных контактов.

Результаты. Дыхательный контроль в КМ интактных животных с возрастом не претерпевал изменений. При СН у 2-месячных крыс ДК не изменялся относительно интактного возрастного контроля. У 15-месячных крыс с СН происходит значимое снижение ДК в КМ как относительно интактных животных этого возраста, так и относительно 2-месячных крыс с СН. Отмечено возраст-зависимое снижение микровязкости мембран КМ в зонах липид-липидных взаимодействий без значимых изменений показателя в местах белок-липидных контактов. При СН у 2-месячных животных микровязкость мембран КМ в зонах белок-липидных взаимодействий существенно понижается, а у 15-месячных повышается относительно интактного контроля в группе. Межгрупповое сравнение выявило возраст-зависимое увеличение микровязкости мембран КМ в области белок-липидных контактов и отсутствие различий в фазе общих липидов.

Заключение. Выявлено отсутствие значимых изменений дыхания с возрастом у интактных крыс. При СН у 15-месячных животных дыхание КМ значительно ниже интактного контроля и 2-месячных животных с СН. Данные изменения могут быть обусловлены различием микровязкостных характеристик мембран клеток в разные периоды онтогенеза.

Ключевые слова: дыхание кардиомиоцитов, микровязкость мембран, возраст-зависимые изменения, крысы

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Соответствие принципам этики. Исследование одобрено комитетом по биомедицинской этике НИИ кардиологии Томского НИМЦ (протокол № 192 от 18.12.2019).

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INTRODUCTION

Heart failure (HF) is a common outcome in a multitude of cardiovascular diseases. According to epidemiological studies, with age, the incidence of HF increases from 3% in the age group of 46–64 years to 10% in the age group of 70 years and older [1]. Recently, a rising incidence of HF in younger adults has been noted. The results of European studies have shown an increase in the proportion of patients under 50 years with new-onset HF [2, 3]. A decrease in respiration in cardiomyocytes (CMs) at later stages of ontogenesis may be one of the possible pathogenetic factors in the development of HF [4]. It is known that the state of a lipid bilayer in biological membranes affects their functioning and the functioning of membrane-bound enzymes [5].

The aim of the study was to compare respiration in CMs and microviscosity characteristics of their membranes in rats of different age groups with HF.

MATERIALS AND METHODS

The research was performed on male Wistar white rats. The animals were divided into 4 groups: 2 groups of intact rats aged 2 and 15 months (n = 12) and 2 groups of animals of similar ages (n = 10) with a model of HF. The isoproterenol-induced HF model was used in the study. Two subcutaneous injections of isoproterenol hydrochloride (Isadrin, Sigma, USA) with an interval of 24 hours (170 mg / kg of rat body weight) were performed. HF developed by day 28 after the second injection [6]. By the time the groups were formed, the weight of 2- and 15-month-old rats averaged 199 (198; 203) and 528 (500; 563) g, respectively. All manipulations on the rats were carried out in compliance with the provisions of the order of the Ministry of Health of the Russian Federation of April 1, 2016 No. 199n "On Approval of the Rules of Good Laboratory Practice". The study was performed within the fundamental research topic of the Cardiology Research Institute, Tomsk NRMC AAAA-A15-115123110026-3. The study was approved by the Bioethics Committee at the Cardiology Research Institute, Tomsk NRMC (Protocol No. 192 of 18.12.2019).

CMs were separated from the isolated heart. The anesthetized rat was submitted to thoracotomy, which provided access to the heart. The excised heart was transferred into ice-cold Krebs – Henseleit solution (mM) (NaCl – 118;KCl – 4.7; KH_2PO_4 – 1.25; $MgSO_4$ – 1.3; $CaCl_2$ – 1.2; glucose – 10) (pH = 7.35–7.40) (Sigma, USA). The connective tissue was removed, isolating the aorta; and the organ was washed from blood. Then it was transferred to the perfusion chamber and perfused via the aorta with oxygenated (95% O_2, 5% CO_2) Krebs – Henseleit solutions with different content of Ca^{2+} ions and proteolytic enzymes (collagenase type II (PanEco, Russia) – 0.2 mg / ml and pronase (Roche Diagnostics, USA) – 0.1 mg / ml). At the end of the perfusion procedure, the enzyme-washed heart was placed in Krebs – Henseleit solution, the ascending aorta was removed, and the heart was cut into 1–2 mm^3 pieces. Then a suspension of isolated CMs was obtained by gentle pipetting [4].

The concentration of total protein in the obtained cell suspension was determined by the micro Lowry assay using a set of Sigma reagents (USA). The optical density in the studied samples was measured by the NanoDrop spectrophotometer (USA) against a reagent blank at λ = 630 nm.

CM respiration was measured on the Expert – 001 fluid analyzer (Ekoniks, Russia) using the Clark DKTP-02.4 sensor immediately after the isolation. An aliquot of cell suspension (protein content of 0.5–1 mg) was placed in the thermostated (t = 25–27 ºC) 1 ml chamber with a pre-oxygenated incubation medium (mM) (sucrose – 0.25; KCl – 10; KH_2PO_4 – 5; $MgCl_2$ – 1.2; succinic acid – 5). Readings for oxygen concentration in the medium after stabilization and "warming" of CM corresponded to the V2 respiration state – free respiration (sufficient amount of oxygen and substrate for oxidation in the medium, but the absence of the phosphorylation substrate – ADP). We measured the rate of oxygen consumption in the medium after adding 100 µl of 0.2 mM ADP solution into it (V3 respiration state) and after its consumption (V4 respiration state). Oxygen consumption by CM was calculated in nM O_2 / min / mg of protein in the sample. The

respiratory control (RC) ratio was calculated by dividing V3 respiration state to V4 [4].

The microviscosity of CM membranes was studied by assessing lateral diffusion in the hydrophobic region of the membranes using pyrene fluorescence and calculating its eximerization coefficients (C_E) $C_E=I_{470}/I_{390}$ at excitation wavelengths of 340 and 285 nm for lipid – lipid and protein – lipid interactions, respectively [7]. The intensity of pyrene dimer formation, characterized by C_E values, was inversely correlated with the membrane microviscosity. The peak luminescence was recorded at 390 nm for pyrene monomers and at 470 nm for pyrene dimers (excimers). The fluorescence of fluorophore was measured on the Cary Eclipse fluorescence spectrometer (Varian, USA).

Statistical processing of the obtained data was performed using the STATISTICA 10.0 software package. Normal distribution of quantitative data was checked using the Shapiro – Wilk test. Non-normal distribution of quantitative data was assessed by the non-parametric Mann – Whitney test. The results were presented as the median and the interquartile range *Me* (Q_1; Q_3). The differences were significant at $p < 0.05$.

RESULTS

Fig. 1 shows the results obtained in the assessment of CM respiration in the studied groups of animals. It can be seen that the groups of intact 2- and 15-month-old animals had no significant differences in the RC coefficient ($p = 0.05$). The value of this coefficient was 3.57 (3.32; 3.93) in 2-month-old rats and 3.36 (3.27; 3.40) in 15-month-old animals.

In the context of developed HF, the studied groups of animals differed significantly ($p < 0.01$) in the RC coefficient. At the same time, it was found that in 2-month-old animals, this coefficient remained virtually unchanged – 3.50 (3.19; 4.34) ($p = 0.86$). On the contrary, in 15-month-old animals with HF, the RC coefficient was significantly lower than in the intact animals of the same age and reached only 2.77 (2.71; 2.78) ($p < 0.05$) (Fig. 1).

When comparing C_E of pyrene fluorescence in areas of protein – lipid interactions in CM membranes of intact animals, no significant ($p = 0.11$) age-dependent differences were found (Fig. 2). On the contrary, when comparing the microviscosity coefficients of CM membranes in the zones of lipid – lipid interactions, an explicit age dependence was established (Fig. 3). In our study, the value of this coefficient in 2-month-old animals was 1.25 (1.01; 1.48), and in 15-month-old animals – 1.73 (1.37; 1.87) ($p < 0.001$).

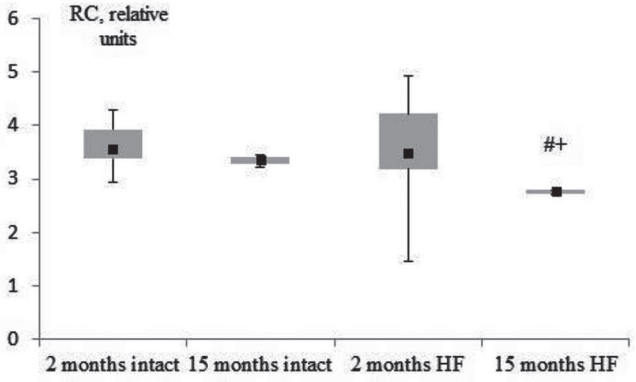

Fig. 1. Respiratory control coefficient (relative units) of rat cardiomyocytes; *Me* (Q_1; Q_3).

RC – respiratory control; HF – heart failure. Significant differences: # between age groups of animals with HF, $p < 0.01$; + between intact and experimental animals within one age group, $p < 0.05$

When comparing C_E of pyrene in the areas of protein – lipid interactions in myocardial cell membranes of animals with HF, a significant age-dependent decrease in this parameter was noted ($p < 0.05$). At the same time, in 2-month-old rats with HF, this coefficient, in comparison with intact animals, was significantly higher and reached 1.21 (1.06; 1.54) ($p < 0.01$). However, in 15-month-old animals with HF, this coefficient was found to be significantly lower and reached 0.88 (0.70; 0.90) ($p < 0.05$) (Fig. 2). In the zones of lipid – lipid interactions, no significant differences in the microviscosity coefficients between intact and experimental animals in both age groups were found.

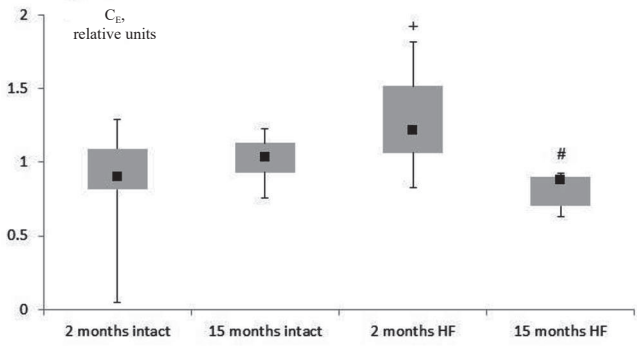

Fig. 2. Cardiomyocyte membrane eximerization coefficient (relative units) in zones of protein – lipid interactions, relative units; *Me* (Q_1; Q_3): C_E – eximerization coefficient; HF – heart failure (here and in Fig. 3); # significant differences between age groups of animals with HF, $p < 0.05$; + significant differences between intact and experimental animals within one age group, $p < 0.01$.

Besides, no significant difference was found in these coefficients between age groups when comparing both subgroups of intact rats and animals with modeled HF (Fig. 3).

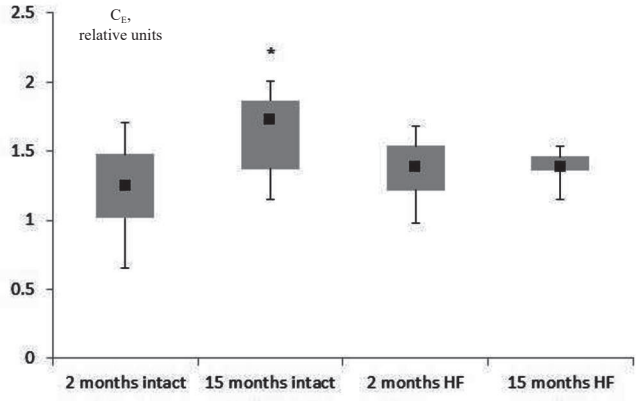

Fig. 3. Cardiomyocyte membrane eximerization coefficient (relative units) in zones of lipid – lipid interactions, relative units; *Me* (Q_1; Q_3): * significant differences between groups of intact 2- and 15-month-old animals, $p < 0.001$

DISCUSSION

The absence of significant differences in CM respiration between the intact animals of both age groups may be due to the activation of adaptive reserve capacities in CMs in adult animals. At the same time, according to the literature data, RC values in both groups fall within the reference norm (3–5 relative units) [8]. Interestingly, in HF in young animals, no significant change in RC was noted, which may indicate greater adaptive reserve capacities in CMs in young animals. In adult rats with HF, this parameter significantly decreased both in young animals with HF and in intact rats of the same age, which may be due to disruption of adaptive responses in cells and a decrease in function with age. It can also be assumed that the injection of toxic doses of isoproterenol increases synthesis of reactive oxygen species (ROS) in the cell in response to the effect of this agent due to intensification of energy production and an increase in electron leakage from the electron transport chain. It is in line with the results of our previous studies, which showed that the activity of antioxidant enzymes in the myocardium decreases with age [9].

The above hypothesis about the change in adaptive reserve capacities of myocardial cells is quite consistent with the data obtained from a comparative analysis of the CM membrane microviscosity in the same groups of animals. The differences in C_E between the groups of intact animals of different ages may be associated with age-dependent changes in cholesterol metabolism. One of the main functions of this neutral lipid is regulation of biological membrane viscosity by changing lateral movement of fatty acid residues in phospholipids [10]. With age, the level of cholesterol in the body increases [11], which may affect its content within the lipid bilayer of the membrane. The latter leads to an increase in the viscosity of membranes, which makes them rigid.

The data obtained in the groups of animals with HF could indicate oppositely directed age-dependent changes in the phospholipid composition of annular lipids, which is reflected in the C_E. These differences may affect the activity of cellular enzyme systems that provide energy metabolism.

CONCLUSION

Therefore, it was found that respiration in isolated CMs of intact animals is not subject to significant age-dependent changes. However, a trend toward its decrease has been noted. This may indicate depletion of compensatory mechanisms in the cell that maintain the optimal respiration level. With the development of HF, these differences become more pronounced, which manifests itself by a significant decrease in respiration in aged rats (15 months old) compared to intact animals of the same age group. At the same time, in 2-month-old rats with HF, the respiration level remains the same as in intact animals.

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The article is devoted to the 75th anniversary of Nikolai A. Kornetov, founder of integrative biomedical (clinical) anthropology, which combines the aspects of morphological and clinical sciences for a clearer understanding of development, clinical presentation, course, and outcomes of a disease

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Constitutional and morphological basis of the metabolic syndrome in patients with schizophrenia and persons without mental disorders

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ABSTRACT

Aim. To identify differences or comparability of constitutional-morphological characteristics and indicators of the fatty constitution between patients with schizophrenia and people with MetS and without mental disorders.

Materials and methods. We examined 63 patients with schizophrenia and MetS (25 women, 38 men), aged 30 [33;52], and 50 mentally healthy individuals with MetS (28 women, 22 men) aged 57 [49; 60]. The main criterion for inclusion in the study was the presence of a verified MetS according to the criteria of the International Diabetes Federation. Anthropometric examination was performed according to the method of V.V. Bunak (1941) with the underlying calculation of integral indices. The determination of the fat component included: measuring waist circumference; non-invasive bioimpedancemetry – body weight, BMI, total and visceral fat content; determination of the total fat fold (electronic caliper). In the blood serum, the concentration of glucose, total cholesterol, HDL, TG was determined using standard commercial kits, the calculation of LDL and the Atherogenic Index.

Results. Differences in the prevalence of the constitutional-morphological type and the type of somatic sexual differentiation were not established in the groups. The level of visceral fat and BMI were higher in mentally healthy individuals with MetS than in schizophrenic patients with MetS ($p = 0.005$ and $p = 0.0001$, respectively). Patients with schizophrenia and MetS had low serum glucose levels compared with individuals without mental disorders ($p = 0.0001$). An increase in the level of TG and the Atherogenic Index was found in patients with schizophrenia with MetS ($p = 0.026$ and $p = 0.03$, respectively), and the level of HDL was reduced ($p = 0.022$).

Conclusion. The constitutional and morphological basis of MetS in patients with schizophrenia and persons without mental disorders is the same, however, changes in the fat constitution were determined for mentally healthy individuals. Changes in the lipid profile and glucose concentration may be associated with the presence of MetS-specific risk factors for patients with schizophrenia.

Keywords: schizophrenia, metabolic syndrome, constitution, BMI, visceral obesity, lipid spectrum.

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the Mental Health Research Institute, Tomsk NRMC (Protocol No. 99 of 17.04.2017).

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Конституционально-морфологические основы метаболического синдрома у пациентов с шизофренией и лиц без психических расстройств

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РЕЗЮМЕ

Цель: выявить различия или сопоставимость конституционально-морфологических характеристик и показателей жировой конституции между пациентами с шизофренией и лицами с метаболическим синдромом (МС) без психических расстройств.

Материалы и методы. Обследованы 63 пациента с шизофренией и МС (25 женщин, 38 мужчин) в возрасте 30 [33; 52] лет и 50 психически здоровых лиц с МС (28 женщин, 22 мужчины) в возрасте 57 [49; 60] лет. Основным критерием включения в исследование являлось наличие верифицированного МС по критериям Международной федерации диабета (IDF). Антропометрическое обследование выполнено по методике В.В. Бунака (1941) с последующим вычислением интегральных индексов. Измерение жировой компоненты включало: проведение измерения окружности талии; неинвазивную биоимпедансометрию – масса тела, индекс массы тела (ИМТ), содержание общего и висцерального жира; определение суммарной жировой складки (электронный калипер). В сыворотке крови определена концентрация глюкозы, общего холестерина, холестерина липопротеидов высокой плотности (ХС-ЛПВП), триглицеридов (ТГ) с использованием стандартных коммерческих наборов, расчет показателей ХС-ЛПНП и индекса атерогенности.

Результаты. Различия в частоте встречаемости конституционально-морфологического типа и типа соматической половой дифференциации не были установлены в группах сравнения. Уровень висцерального жира и ИМТ были значительно выше у психически здоровых лиц с МС, чем больных шизофренией с МС ($p = 0{,}005$ и $p = 0{,}0001$ соответственно). Пациенты с шизофренией и МС имели низкий уровень концентрации глюкозы в сыворотке крови по сравнению с лицами без психических расстройств ($p = 0{,}0001$). Обнаружено повышение уровня ТГ и индекса атерогенности у больных шизофренией с МС ($p = 0{,}026$ и $p = 0{,}03$ соответственно), а уровень ХС-ЛПВП был снижен ($p = 0{,}022$).

Заключение. Конституционально-морфологическая основа формирования МС у больных шизофренией и лиц без психических расстройств является одинаковой, однако изменения в жировой конституции были определены для психически здоровых лиц. Изменения в липидном профиле и концентрации глюкозы могут быть связаны с наличием специфичных для больных шизофренией факторов риска МС.

Ключевые слова: шизофрения, метаболический синдром, конституция, ИМТ, висцеральное ожирение, липидный спектр

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при финансовой поддержке РНФ в рамках научного проекта № 18–15–00011.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом НИИ психического здоровья Томского НИМЦ (протокол № 99 от 17.04.2017).

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INTRODUCTION

The development of metabolic syndrome (MetS) increases the risk of developing cardiovascular diseases, insulin resistance, diabetes mellitus, as well as vascular and neurological complications [1]. MetS is associated with the risk of death for mentally healthy individuals [2], for patients with schizophrenia, it is increased by almost 2–4 times [3].

Antipsychotic therapy is a factor for the development of MetS in patients with schizophrenia and an increase in the deterioration of the metabolic profile in the first two years of therapy, especially for younger patients and for individuals with the first episode [4]. Although various criteria for diagnosing MetS are used, the prevalence of MetS increases over time in the age group from 20 to 29 years: according to ATP III A criteria the increase is from 38.9 to 53.0% and according to IDF, it is from 43.6 to 55.7% [5].

Treatment with conventional or atypical antipsychotics is associated to varying degrees with the incidence of metabolic disorders. In this series, quetiapine, olanzapine, and clozapinum can be distinguished as antipsychotics with the highest risk of developing MetS, while aripiprazole and haloperidol have a low risk [6].

Individual components of the fat constitution, namely, an increase in waist circumference parameters and body mass index (BMI), are associated with the risk of developing MetS in patients with schizophrenia [7]. Also, an increase in subcutaneous and visceral fat was found during treatment with antipsychotics, while the total body fat percentage did not increase in patients with schizophrenia compared with healthy controls [8].

Previously, constitution as a structural biomarker was found to have a crucial role in the development of visceral obesity in patients with schizophrenia while they were treated with antipsychotics [9]. The asthenic constitutional and morphological type was identified as a risk factor for the development of MetS in patients receiving risperidone, and the type of somatic sexual differentiation was identified for quetiapine. This was an extended application of the anthropological method in psychiatry, which was traditionally used in relation to the clinical presentation and the course of mental disorders [10] to assess a response to therapy and its safety. Some studies also demonstrated the association of anthropometric parameters including an increase in BMI and waist-to-hip ratio with the risk of sudden cardiac death, which was explained by the development of metabolic disorders in mentally healthy population [11].

The study of the relationship between epicardial fat, obesity, visceral fat, and MetS components showed the contribution of the components to the risk of developing coronary heart disease [12]. Schizophrenia patients were found to have a higher incidence of abdominal obesity, hypertriglyceridemia, and a decrease in the level of high-density lipoproteins, while there was a slight decrease in the incidence of hyperglycemia when compared with mentally healthy individuals [13]. The predictive value of high-density lipoprotein cholesterol (HDL-C) in relation to MetS was recognized as the highest in patients with schizophrenia receiving antipsychotic therapy [14]. It should be noted that MetS has a complex and systemic pathogenesis, however, along with other possible predictors, sedentary lifestyle and unbalanced diet play a fundamental role in its development [15].

Studies demonstrate the contribution of individual anthropometric indicators to the development of MetS in both patients with schizophrenia and individuals without mental disorders. Previously, no studies were conducted aimed at comparing constitutional factors in the formation of MetS between these two groups.

The aim of the study was to identify differences or comparability of constitutional and morphological characteristics and indicators of the fat constitution between patients with schizophrenia and people with MetS without mental disorders.

MATERIALS AND METHODS

The study was carried out in the clinics of the Mental Health Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences (second clinical department) and Siberian State Medical University (endocrinology department). All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the Mental Health Research Institute of Tomsk NRMC (Protocol No. 99 of 17.04.2017).

Two study groups were formed: patients with schizophrenia and mentally healthy individuals with MetS. The main criterion for inclusion in the study was the presence of a verified MetS diagnosis according to the criteria of the International Diabetes Federation (IDF) (2005) [16], which include the presence of abdominal obesity (waist circumference ≥ 94 cm in men and ≥ 80 cm in women) accompanied by 2 or more of the following factors: an increase in triglycerides ≥ 1.7 mmol / l or receiving specific treatment for this dyslipidemia; cholesterol ≤ 1.03 mmol / l in men and 1.29 mmol / l in women; a decrease in HDL or receiving specific treatment for this dyslipidemia; increased blood pressure ≥ 130 / 85 mm Hg or receiving antihypertensive therapy; an increase in fasting blood glucose ≥ 5.6 mmol / l or the previous diagnosis of type 2 diabetes mellitus. Criteria for inclusion in the study were as follows: the age of patients from 18 to 60 years, a verified diagnosis of schizophrenia according to the criteria for studies according to ICD-10 [17] (for patients from a psychiatric hospital), belonging to the Caucasian race, and an ability to give a written informed consent. Exclusion criteria were the presence of organic, neurological, and severe somatic disorders leading to organ failure, and refusal to participate in the study. All patients with schizophrenia at the time of inclusion in the study received basic antipsychotic therapy at therapeutic doses approved by the Ministry of Healthcare of the Russian Federation.

An anthropometric examination was performed according to the method of V.V. Bunak (1941) [18], adopted at the Research Institute and the Museum of Anthropology named after D.N. Anuchin of Lomonosov Moscow State University. The determination of constitutional and morphological types and somatic sexual differentiation was carried out using the calculation of the Rees-Eysenck Body Index [19] and the Tanner scale [20].

The measurement of the fat component included measuring waist circumference (measuring tape); non-invasive bioimpedancemetry (medical device "Omron BF508", Japan) – body weight, BMI, total and visceral fat; total fat fold (electronic caliper) which consisted of the sum of the fat fold values of the shoulder, back, abdomen, and lower leg.

Blood sampling was carried out after a 12-hour overnight fasting by antecubital venipuncture into clot activator tubes (CAT; BD Vacutainer). To isolate serum samples, the tubes were centrifuged for 30 min at 2,000 g at 4 °C. Serum was stored at –20 °C (or –80 °C) before the analysis. The concentration of total cholesterol (TC), HDL, triglycerides (TG), and glucose in blood serum was determined by the enzymatic colorimetric method using standard commercial kits (Cormay, Poland). Low-density lipoprotein (LDL) concentrations were calculated using the Friedewald equation (1972) [21]. The atherogenic index was calculated according to the formula proposed by A.N. Klimov (1977) [22].

The obtained data were tested for normal distribution using the Kolmogorov – Smirnov test (with the Lilliefors correction) and the Shapiro – Wilk test. Quantitative data were presented as the median and the interquartile range Me [Q_1; Q_3]. Qualitative data were presented by frequency indicators (n (%)). A statistical analysis was performed using the Statistica software for Windows 12.0. Pearson's $\chi 2$ test was used to compare the frequencies. The Mann – Whitney U-test was used to compare two independent samples of quantitative data. The threshold value of the achieved significance level was $p \leq 0.05$.

RESULTS

We examined 63 schizophrenia patients with MetS (25 women, 38 men) aged 30 [33; 52] years and 50 mentally healthy individuals with MetS (28 women, 22 men) aged 57 [49; 60] years. The study groups were comparable by sex (p = 0.084), however, mentally healthy individuals with MetS were significantly older than schizophrenia patients (p = 0.0001).

The groups were compared according to the frequency of occurrence of the constitutional and morphological type. We did not establish statistically significant differences between patients with schizophrenia and mentally healthy individuals with MetS (Table 1). In both groups, persons with the andromorphic type prevailed – 65.1% and 56.0%, respectively.

Table 1

Constitutional and morphological types in the study groups

Indicators	Patients with schizophrenia and MetS		Mentally healthy individuals with MetS		*p*
	Abs.	%	Abs.	%	
Asthenic	7	11.1	0	0.0	–
Mesomorph	35	55.6	24	48.0	0.453
Hypersthenic	21	33.3	26	52.0	0.055
Total	63	100.0	50	100.0	–

In relation to the type of somatic sexual differentiation, differences were not established in both study groups ($p = 0.565$) and pairwise comparison (Table 2).

Table 2

Type of somatic sexual differentiation in the study groups

Indicators	Abs.	%	Abs.	%	*p*
Andromorphic	41	65.1	28	56.0	0.339
Mesomorph	9	30.2	18	36.0	0.549
Gynecomorphic	3	4.8	4	8.0	0.697
Total	63	100.0	50	100.0	–

Comparison of indicators of the fat component in body composition is presented in Table 3. It was found that BMI and visceral fat levels were significantly higher in mentally healthy individuals with MetS than in patients with schizophrenia and MetS ($p = 0.005$ and $p = 0.0001$, respectively). This may be due to their older age and, as a result, longer duration of obesity and the risk of developing insulin resistance and prediabetes.

Table 3

Indicators of the fat component in the body composition in patients with schizophrenia and mentally healthy people with metabolic syndrome, *Me* [Q_1; Q_3]

Indicators	Patients with schizophrenia and MetS	Mentally healthy individuals with MetS	*p*
Body weight, kg	95.0 [84.2; 105.7]	95.7 [89.1; 106.5]	0.164
Waist, cm	105.0 [96.5; 114.0]	109 [101; 115]	0.205
BMI	31.2 [27.5; 35.9]	33.7 [31.5; 38.3]	0.005
Total body fat	36.4 [30.5; 47.5]	40.8 [33.7; 48.4]	0.176
Visceral fat level	11 [9; 14]	13 [11; 17]	0.0001
Total fat fold	116 [86; 135]	121 [101; 143]	0.167

It was found that patients with schizophrenia and MetS had a low level of glucose in blood serum compared to mentally healthy individuals with MetS, which was statistically significant ($p = 0.0001$). The increase in lipid spectrum indicators for the level of TG and the atherogenic index was revealed in patients with schizophrenia and MetS ($p = 0.026$ and $p = 0.03$, respectively), and the level of HDL-C was reduced ($p = 0.022$) (Table 4).

Table 4

Glucose and lipid profile in patients with schizophrenia and mentally healthy people with metabolic syndrome, mmol / l, *Me* [Q_1; Q_3]

Indicators	Patients with schizophrenia and MetS	Mentally healthy individuals with MetS	*p*
Glucose	5.20 [4.70; 5.85]	5.70 [5.40; 6.40]	0.0001
TC	5.00 [4.04; 5.61]	4.66 [3.83; 5.62]	0.783
TG	2.00 [1.74; 2.41]	1.85 [1.50; 2.10]	0.026
HDL-C	0.90 [0.70; 1.10]	1.00 [0.90; 1.20]	0.022
LDL	2.92 [2.05; 3.72]	2.90 [2.10; 3.92]	0.804
Atherogenic index	4.26 [3.42; 5.98]	3.67 [2.67; 4.50]	0.030

DISCUSSION

The results obtained suggest that the constitutional and morphological basis for the formation of MetS in patients with schizophrenia and in mentally healthy individuals is the same. The results are of fundamental importance, since they confirm the general pathophysiology of MetS [23] in different groups of patients and the role of the bone component of the constitution as a time-stable structural marker in the mechanisms of MetS development. This indicates that the constitution affects the risk of developing MetS to a greater extent than antipsychotic drugs and schizophrenia itself as a pathological process accompanied by immune inflammation, even though their significant contribution to MetS is well known [24, 25].

However, differences were found in the fat constitution, since such indicators as BMI and visceral fat level increased in mentally healthy individuals with MetS compared with patients with schizophrenia and MetS. Previously, it was shown that the determination of BMI has a prognostic value for clinical screening of metabolic disorders in patients with schizophrenia, namely, persons whose BMI was 28 kg / m^2 and above had a higher risk of developing MetS than persons with an indicator below this threshold value [26].

Also, the literature provides evidence that for mentally healthy individuals, the level of BMI is not defined as a possible marker for the prognosis of metabolic disorders [27].

Currently, one of the key pathophysiological factors in the onset of MetS is an increase in the visceral fat [15], it is believed that this parameter can be a reliable predictor of the risk of developing metabolic disorders [28]. There is also an opposite point of view, where the study did not establish differences in the distribution of visceral fat in patients with schizophrenia receiving antipsychotic therapy and the control group according to magnetic resonance imaging (MRI) [29].

Changes in the metabolism of glucose and lipids in patients with schizophrenia are observed after 2 weeks and reach a maximum after 3 months while receiving antipsychotic therapy [30]. It was determined that fasting plasma glucose, insulin, and glucose in an oral glucose tolerance test increased and insulin resistance formed in patients with the first episode of schizophrenia, previously not treated with neuroleptics, compared with mentally healthy individuals, which may indicate the development of glucose metabolism disorders in patients before therapy [31].

In this study, individuals with schizophrenia and MetS had low serum glucose level. Glucose level increased in mentally healthy individuals with MetS, which is consistent with an increase in indicators such as BMI and visceral fat level in this group. Impaired glucose metabolism reflects the formation of insulin resistance, which leads to the development of visceral obesity, dyslipidemia, and its progression [32].

In the first 6 months of antipsychotic therapy in patients with schizophrenia, a correlation was found between the development of metabolic disorders in the form of dyslipidemia with low HDL levels and an increase in waist circumference [33]. The effect of a pharmacological agent on neurohumoral systems, namely the metabolism of leptin, adiponectin, ghrelin, orexin and cholecystokinin, dopaminergic, serotonergic, adrenergic, and histamine receptors, which are considered to be biomarkers of metabolic disorders, is associated with the formation of MetS [34], an increase in TC, TG, and LDL-C, and a decrease in HDL-C in patients with schizophrenia [35].

The revealed differences in the age of patients in the comparison groups can be explained by the fact that patients were included in the study at the same time, which was determined by the design of the study. It can be assumed that the older age of inpatients with MetS reflects the general picture of MetS development in the general population, where the overall prevalence of MetS increases with age: 15–39 years – 13.9%; 40–59 years – 26.4%, > 60 years – 32.4% [36]. Previously, gender and age differences were also shown for patients with schizophrenia, namely, women with MetS were older, had longer duration of MetS, and developed it later than men with MetS [37].

The new results obtained in this study are considered from interdisciplinary perspective, which is widely used to assess mental health [38]. In this case, the application of the anthropological approach in psychoendocrinology made it possible to find clinical and biological similarities and differences in the characteristics of MetS in patients with schizophrenia and individuals without mental disorders.

CONCLUSION

The comparative analysis of constitutional and morphological characteristics in the groups of patients with schizophrenia and MetS and mentally healthy individuals with MetS did not show significant differences in the constitutional and morphological type or the type of somatic sexual differentiation. In relation to the fat constitution, it was found that BMI and the level of visceral fat were higher in mentally healthy individuals with MetS than in patients with schizophrenia. The analysis of the level of glucose and lipid profile in the study groups revealed that serum glucose and HDL-C levels were reduced, while TG and the atherogenic index were increased in patients with schizophrenia and MetS. The revealed changes can probably be associated with the presence of MetS risk factors specific to patients with schizophrenia.

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Kornetova E.G. – conception and design, drafting of the manuscript, critical revision of the manuscript for important intellectual content. Goncharova A.A. – clinical, psychopathological, and anthropometric survey of the sample, statistical processing of the data, review of the literature on the research topic, drafting of the manuscript. Mednova I.A. – biochemical examination, review of the literature on the research topic, drafting of the manuscript. Kornetov A.N. – drafting of the manuscript, critical revision of the manuscript for important intellectual content. Saprina T.V. – critical revision of the manuscript for important intellectual content. Perchatkina O.E. – clinical, psychopathological, and anthropometric survey of the sample. Semke A.V. – final approval of the manuscript for publication.

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Diagnosis of sentinel lymph nodes in patients with cancer of the larynx and laryngopharynx using a new radiopharmaceutical based on technetium-99m-labeled gamma aluminum oxide

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ABSTRACT

Aim. To study the possibility of using a radiopharmaceutical based on aluminum oxide labeled with ^{99m}Tc ([^{99m}Tc]-Al_2O_3) for the diagnosis of sentinel lymph nodes (SLN) in tumors of the larynx and laryngopharynx in comparison with a phytate colloid ([^{99m}Tc]-phytate colloid).

Materials and methods. The study included patients with cancer of the larynx and laryngopharynx ($T_{2-4}N_0M_0$) (n = 54). In the prospective group (*n* = 30), [^{99m}Tc]-Al_2O_3 was used as a radiopharmaceutical, in the retrospective group (n = 24), [^{99m}Tc]-phytate colloid was used. All radiopharmaceuticals were introduced endoscopically into the submucosal space along the periphery of the tumor. After 18 hours, single-photon emission computed tomography (SPECT) and intraoperative SLN detection were performed.

Results. In the retrospective group, SLNs were detected in 20 out of 24 patients. A total of 32 lymph nodes were identified in the retrospective group. The median number of detected lymph nodes in one patient was 1.3 [0–3], the intensity of the radiopharmaceutical uptake on scintigrams was 2.2 [0.7–8.1], intraoperatively – 4 [1.6–9.0]. In the prospective group, [^{99m}Tc]-Al_2O_3 uptake in the lymph nodes of the neck was determined in 27 patients (90%); in 3 patients, SLNs were not visualized. A total of 57 lymph nodes were identified (in 27 patients). The median number of visualized SLNs was 1.5 [0–5], the intensity of [^{99m}Tc]-Al_2O_3 uptake according to SPECT and intraoperative detection was 4.8 [0.7–19.4] and 6 [1.1–22.0], respectively.

Conclusion. The most significant advantage of using [^{99m}Tc]-Al_2O_3 as a radiopharmaceutical is its high uptake in SLNs, which leads to an increase in the sensitivity of the method as a whole up to 90 versus 83% when using [^{99m}Tc]-phytate colloid.

Keywords: radionuclide, colloid, laryngeal cancer, laryngopharyngeal cancer, sentinel lymph node, single-photon emission computed tomography, gamma probe

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee and the Bioethics Committee at Cancer Research Institute, Tomsk NRMC.

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Диагностика сторожевых лимфатических узлов у больных раком гортани и гортаноглотки с применением нового отечественного радиофармацевтического лекарственного препарата на основе меченного технецием-99m гамма-оксида алюминия

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РЕЗЮМЕ

Цель. Изучить возможность использования радиофармацевтического лекарственного препарата (РФЛП) на основе оксида алюминия, меченного ^{99m}Tc ([^{99m}Tc]-Al_2O_3), для диагностики сторожевых лимфатических узлов (СЛУ) при опухолях гортани и гортаноглотки в сравнении с фитатным коллоидом ([^{99m}Tc]-фитатный коллоид).

Материалы и методы. В исследование вошли больные раком гортани и гортаноглотки стадий $T_{2–4}N_0M_0$ (n = 54). В проспективной группе (n = 30) в качестве диагностического РФЛП использовался [^{99m}Tc]-Al_2O_3, в ретроспективной группе (n = 24) – [^{99m}Tc]-фитатный коллоид. Все РФЛП вводились эндоскопически в подслизистое пространство по периферии опухоли, через 18 ч проводилась однофотонная эмиссионная компьютерная томография (ОФЭКТ) и интраоперационная детекция СЛУ.

Результаты. В ретроспективной группе СЛУ были выявлены у 20 пациентов из 24. Всего в ретроспективной группе было обнаружено 32 лимфатических узла. Количество выявленных лимфатических узлов у одного больного составило 1,3 [0–3], интенсивность накопления РФЛП на томосцинтиграммах – 2,2 [0,7–8,1], интраоперационно – 4 [1,6–9,0]. В проспективной группе аккумуляция [^{99m}Tc]-Al_2O_3 в лимфатических узлах шеи определялась у 27 пациентов (90%), у 3 пациентов СЛУ не визуализировались. Всего было выявлено 57 лимфоузлов (у 27 пациентов). Количества визуализируемых СЛУ составила 1,5 [0–5], интенсивность накопления [^{99m}Tc]-Al_2O_3 по данным ОФЭКТ и интраоперационной детекции составила 4,8 [0,7–19,4] и 6 [1,1–22,0] соответственно.

Заключение. Существенным достоинством использования в качестве РФЛП [^{99m}Tc]-Al_2O_3 является его высокая аккумуляция в сторожевых лимфатических узлах при опухолях гортани и гортаноглотки, что приводит к увеличению чувствительности метода в целом до 90 против 83% при применении [^{99m}Tc]-фитатного коллоида.

Ключевые слова: радионуклидный, коллоид, рак гортани, рак гортаноглотки, сторожевой лимфатический узел, однофотонная эмиссионная компьютерная томография, гамма-зонд

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом по биомедицинской этике НИИ онкологии Томского НИМЦ.

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INTRODUCTION

Surgery is one of the main stages in the treatment of tumors of most localizations. However, surgical intervention often causes a decrease in the quality of life and social adaptation of patients. In this regard, introducing organ-preserving and reconstructive surgeries into clinical practice is extremely relevant. As a result, correct determination of the spread of the malignant process plays an important role in the choice of treatment methods for cancer patients [1–4]. Currently, the sentinel lymph node (SLN) status is considered one of the important prognostic factors for metastasis of some tumors [5–8]. SLN is a lymph node that directly receives lymphatic drainage from a tumor site. R.M. Cabañas was one of the first to coin the term "sentinel lymph node" [9].

Radionuclide diagnosis is actively used in oncological practice, supplementing conventional imaging methods with valuable functional information [10–14] The use of this method for SLN detection makes it possible to accurately localize SLN both intra- and preoperatively. In 1993, J.C. Alex and D.N. Krag suggested to use intraoperative detection of SLN with a portable gamma-ray scanner by measuring the level of radiation in all lymphatic collectors after administration of a radioactive colloid [15].

In recent years, rapid development of nuclear medicine has been associated not only with technological advancements, but also with introduction of new highly specific radiopharmaceuticals. An ideal radiopharmaceutical for SLN imaging should be characterized by rapid clearance from the injection site and active uptake in the lymph nodes. Researchers believe that such characteristics can be encompassed in the radiopharmaceuticals which are based on ligand – receptor or antigen – antibody binding mechanisms. One of such radiopharmaceuticals is currently [^{99m}Tc]-rituximab, which binds to the CD20 receptor, actively expressed on the surface of B cells. Studies have shown that [^{99m}Tc]-rituximab is to a lesser extent redistributed to the distal lymph nodes, compared with colloidal radiopharmaceuticals, and more actively leaves the injection site [16]. Besides, 99mTc-labeled Lymphoseek™ was registered in the United States and is being actively introduced into the clinical practice. This radiopharmaceutical accumulates in the lymph nodes by binding to CD206 on the surface of macrophages [17].

Despite the emergence of targeted radiopharmaceuticals for SLN mapping, research is ongoing to explore the possibilities of using 99mTc-labeled colloidal preparations which are still the main indicators for SLN visualization. The main criterion for assessing a colloidal radiopharmaceutical is the particle size. A colloid with a particle size of 50–80 nm is considered optimal because it provides fast radiopharmaceutical clearance from the injection site and its high retention in the SLN [18, 19].

The aim of this study was to investigate the possibilities of using a new radiopharmaceutical based on aluminum oxide labeled with ^{99m}Tc ([^{99m}Tc]-Al_2O_3) for the diagnosis of SLN in tumors of the larynx and laryngopharynx in comparison with a phytate colloid ([^{99m}Tc]- phytate colloid) commonly used in Russia.

Both radiopharmaceuticals are colloidal preparations, however, they differ in particle sizes: [^{99m}Tc]-phytate colloid is characterized by a fairly large range of particle sizes – 40–10.000 nm, and [^{99m}Tc]-Al_2O_3 contains colloidal particles ranging from 50 to 100 nm in size. Preclinical and clinical trials of [^{99m}Tc]-Al_2O_3 have shown its safety and functional suitability for imaging of lymph nodes [20–25].

MATERIALS AND METHODS

The study included patients with cancer of the larynx and laryngopharynx $T_{2-4}N_0M_0$ (n = 54). The general inclusion criterion was the absence of signs of metastasis in regional lymph nodes at the time of the examination. The study was approved by the local Ethics Committee and the Bioethics Committee at Cancer Research Institute, Tomsk NRMC. All patients signed an informed consent to participate in the study.

In the prospective group (n = 30), [^{99m}Tc]-Al_2O_3 was used as a diagnostic radiopharmaceutical, the retrospective group (n = 24) was examined using [^{99m}Tc]-phytate colloid. The result of a histologic examination of removed lymph nodes was the reference method for analyzing the diagnostic effectiveness.

Radionuclide diagnosis of SLN includes injection of radiopharmaceuticals, visualization, and intraoperative detection of lymph nodes with their subsequent morphological examination. Radiopharmaceuticals were injected endoscopically into the submucosal space along the periphery of the tumor in the region of the larynx or laryngopharynx at 2 sites (at 12:00 and 18:00 on the conventional dial). The total administered radiopharmaceutical activity was 40 MBq (20 MBq per injection site).

Single-photon emission computed tomography (SPECT) was performed on the E.cam 180 gamma camera (Siemens, Germany) 18–20 hours after the

radiopharmaceutical injection using parallel-hole high-resolution collimators for 140 keV radiation..

SPECT images were assessed visually (identifying the number of areas with focal radiopharmaceutical uptake corresponding to the localization of the regional lymph nodes) and semi-quantitatively (calculating the ratio of the number of impulses in the lymph node with the highest radiopharmaceutical uptake to the number of impulses at the injection site, %).

During the surgical intervention, intraoperative SLN detection was performed using the Gamma Finder II probe (USA) (Fig. 1). The lymph node with the highest activity, in comparison with other lymph nodes detected by the gamma probe, or the only lymph node detected, was marked as SLN. After removal of the SLN, the area of the lymphatic collector was re-examined using the gamma probe.

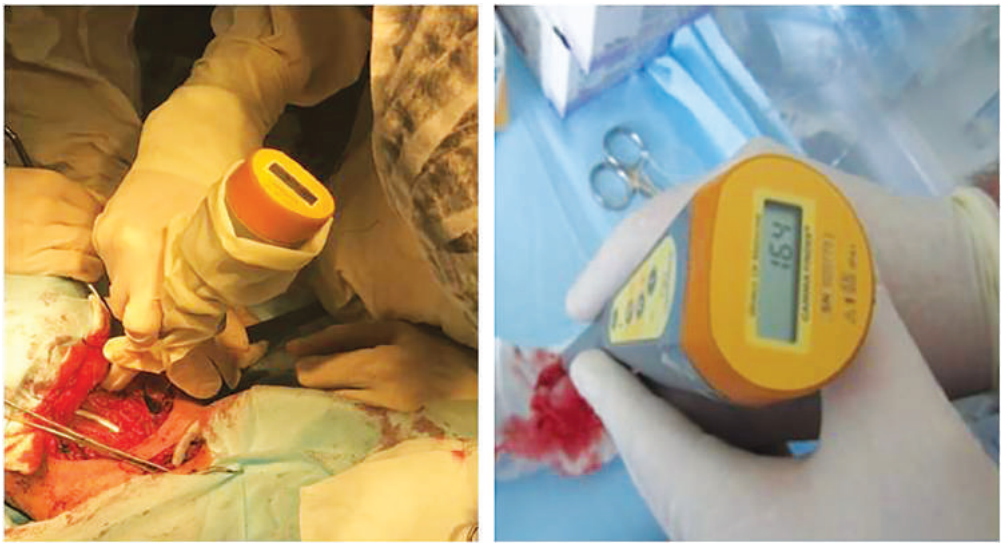

Fig. 1. Intraoperative assessment of sentinel lymph nodes using a gamma probe

All lymph nodes identified by the gamma probe in the projection of the lymphatic collector were removed. According to intraoperative radiometry, the level of radiopharmaceutical uptake in the projection of SLN was also calculated (in % relative to the injection site). Removed SLNs were subject to immediate cytological examination: with metastasis in the lymph nodes, lymph node dissection was performed.

The normality of data distribution was assessed using the Shapiro –Wilk test. The median and the interquartile range *Me* [Q_1 – Q_3] were calculated to measure the central tendency in the sample. The Mann – Whitney *U* test and the Kruskal – Wallis test were used to compare the intergroup differences in the studied results for quantitative variables with non-normal distribution. The sensitivity of the method was assessed according to the formula TP / (TP + FN) × 100%, where TP is a true positive result, and FN is a false negative result. At the same time, we took into consideration the number of patients with radiopharmaceutical uptake in the projection of the lymph nodes. The reference method for the analysis was the result of the histologic examination of the surgical material (the presence of lymph nodes in the removed material).

RESULTS

In 7 out of 54 patients, no redistribution of the radiopharmaceuticals from the injection site along the lymphatic collector was noted. No radiopharmaceutical uptake in the projection of the lymph nodes was detected either scintigraphically or intraoperatively using the gamma probe. Unilateral localization of SLN was visualized in 85% of cases ($n = 40$), bilateral localization was detected in 7 patients (Fig. 2, 3). At the same time, most often lymph nodes were visualized in the projection of levels III and Va of the neck – 47 and 33%, respectively, less often – in the projection of levels IIa and IIb of the neck – 14 and 12%, respectively.

In the retrospective group, SLNs were detected in 20 out of 24 patients. The results of SPECT and intraoperative detection were in line; all lymph nodes visualized by SPECT were also detected by the gamma probe. The presence of [^{99m}Tc]-phytate colloid in the lymph nodes was not observed in 4 patients (Table 1).

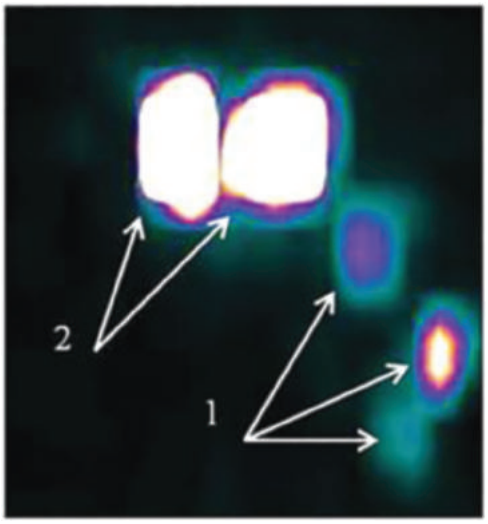

Fig. 2. SPECT image of a patient with laryngeal cancer: 1 – radiopharmaceutical uptake in the projection of the lymph nodes of the neck on the left (n = 3); 2 – injection site

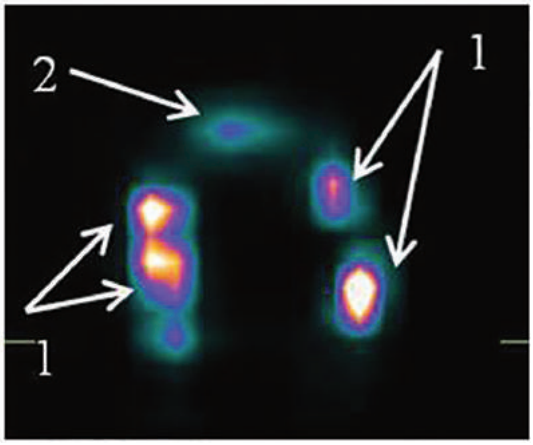

Fig. 3. SPECT image of a patient with laryngopharyngeal cancer: 1– radiopharmaceutical uptake in the projection of the lymph nodes of the neck on both sides (n = 5); 2 – injection site

Table 1

Characterization of the results obtained in the retrospective group using [^{99m}Tc]-phytate colloid, n = 24

Result	SPECT, n	Intraoperative detection, n
True positive	20	20
True negative	0	0
False positive	0	0
False negative	4	4

A total of 32 lymph nodes were identified in the retrospective group. The median number of detected lymph nodes in one patient was 1.3 [0–3], the intensity of the radiopharmaceutical uptake according to SPECT and intraoperative detection was 2.2 [0.7–8.1] and 4 [1.6–9], respectively (Table 2).

In the prospective group, [^{99m}Tc]-Al_2O_3 uptake in the lymph nodes of the neck was determined in 27 patients (90%); SLNs were not visualized in 3 patients. Identical results were obtained during intraoperative detection (Table 3). A total of 57 lymph nodes were identified (in 27 patients). The median number of visualized SLNs was 1.5 [0–5], the intensity of [^{99m}Tc]-Al_2O_3 uptake according to SPECT and intraoperative detection was 4.8 [0.7–19.4] and 6 [1.1–22], respectively (Table 2).

Table 2

Comparative analysis of the results of radionuclide diagnosis of sentinel lymph nodes in patients with cancer of the larynx and laryngopharynx using [^{99m}Tc]-phytate colloid and [^{99m}Tc]-Al_2O_3

Parameter	[^{99m}Tc]-phytate colloid	[^{99m}Tc]-Al_2O_3
Number of visualized lymph nodes, n, Me [Q_1 – Q_3]	1.3 [0–3]	1.5 [0–5]
	$p < 0.2$	
Intensity of radiopharmaceutical uptake according to SPECT, %, Me [Q_1 – Q_3]	2.2 [0.7–8.1]	4.8 [0.7–19.4]
	$p < 0.027$	
Intensity of radiopharmaceutical uptake according to intraoperative detection, %, Me [Q_1 – Q_3]	4 [1.6–9]	6 [1.1–22]
	$p < 0.034$	
Method sensitivity, %	83	90

Table 3

Characterization of the results obtained in the prospective group using [^{99m}Tc]-Al_2O_3, n = 30

Result	SPECT, n	Intraoperative detection, n
True positive	27	27
True negative	0	0
False positive	0	0
False negative	3	3

According to the results of the histologic examination of the removed lymph nodes, metastasis was detected in 3 patients from the retrospective group and in 3 individuals from the prospective group (all affected lymph nodes corresponded to the side of the primary tumor localization).

DISCUSSION

The comparative analysis of the results obtained indicated significant differences in the nature of the scintigraphic data obtained after the use of radiopharmaceuticals with different sizes of colloidal particles. The most significant advantage of using [^{99m}Tc]-Al_2O_3 is its high uptake in the lymph nodes, which improves visualization of SLNs, facilitates their search during surgery, and increases the sensitivity of the method as a whole up to 90 versus 83% when using [^{99m}Tc]-phytate colloid.

To date, surgery remains the main method for treating cancer of the larynx and laryngopharynx. The volume of surgery is determined by clinical and morphological features of the tumor spread. One of the basic factors that affects the treatment strategy and the prognosis of the disease is assessment of the SLN status. In cancer of the larynx and laryngopharynx, the frequency of metastasis to regional lymph nodes reaches 30% [26].

Many attempts have been made to identify predictors of latent metastases in the lymph nodes, of which the depth of invasion turned out to be the most informative. However, this criterion does not provide absolute confidence in the adequate assessment of the state of the regional lymphatic drainage zones [27]. Therefore, application of the concept of SLNs in tumors of the larynx and laryngopharynx can help reduce unnecessary intraoperative lymphadenectomy and decrease the amount of radiation therapy performed [28–30].

In general, methods for determining SLNs in malignant tumors of the head and neck are more related to oral cavity cancer, and the bulk of the research is devoted to this localization. This is due to a more aggressive course of this pathology, features of lymphatic drainage, and difficulties in detecting metastasis in regional lymph nodes, especially at early stages of the disease. Cancer of the larynx and laryngopharynx is characterized by a lower risk of metastasis to the lymph nodes, however, the problem of organ-preserving surgeries in this localization is quite dire. Therefore, developing methods for proper assessment of tumor spread at the preoperative stage is highly relevant [28, 30–32].

CONCLUSION

A comparative study of the effectiveness of [^{99m}Tc]-Al_2O_3 for visualization of SLN in tumors of the larynx and laryngopharynx in comparison with [^{99m}Tc]-phytate colloid showed that a significant advantage of using [^{99m}Tc]-Al_2O_3 is its higher uptake in SLNs. This phenomenon contributes to an increase in the sensitivity of the method as a whole up to 90 versus 83% when using [^{99m}Tc]-phytate colloid.

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Authors contribution

Medvedeva A.A., Chernov V.I., Choynzonov E.L. – conception and design, analysis and interpretation of the data, justification of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Bragina O.D., Zeltchan R.V., Chizhevskaya S.Yu., Rybina A.N., Gol'dberg A.V., Cheremisina O.V. – justification of the manuscript and critical revision of the manuscript for important intellectual content.

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Morphological changes in the myocardium of rats with chronic alcohol intoxication after treatment with new GABA- and glutamic acid derivatives

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ABSTRACT

Aim. To study pathohistological changes in the myocardium of rats with chronic alcohol intoxication (CAI) after treatment with a new glutamic acid derivative glufimet (compound RSPU-238) and a new gamma-aminobutyric acid (GABA) derivative (compound RSPU-260).

Materials and methods. Experiments were performed on female Wistar rats aged 10 months. The rats were divided into the following groups: group 1 – intact females; group 2 – a control group which included animals after CAI simulated by replacing drinking water with 10% ethanol solution for 24 weeks; groups 3 and 4 – experimental groups, in which females were intraperitoneally administered with glufimet at a dose of 28.7 mg / kg and RSPU-260 at a dose of 25 mg / kg once a day for 14 days after cessation of alcohol solution consumption; group 5 – a group of animals receiving a reference listed drug mildronate at a dose of 50 mg / kg according to a regimen similar to that of the studied compounds. Changes in microstructural and morphometric parameters of the left ventricular myocardium were assessed using light microscopy.

Results. In animals after CAI, the cardiomyocyte volume fraction decreased, while the interstitial and vascular volume fractions increased. Degeneration of cardiomyocytes, such as their wave-like deformation, loss of transverse striation, foci of plasmolysis, and fragmentation of muscle fibers were revealed. In rats treated with glufimet, the structural changes in cardiomyocytes were minimal. Lower vascular plethora was observed; blood vessels were characterized by single stasis and sludge. The cardiomyocyte volume fraction was 9.7% greater than in control animals, while the interstitial and vascular volume fractions were 66.0 and 70.0% smaller, respectively. The animals treated with the RSPU-260 compound had no significant degenerative changes in cardiomyocytes and small vessels similar to the experimental animals injected with glufimet. Mildronate had a less pronounced cardioprotective effect.

Conclusion. Administration of new GABA and glutamic acid derivatives to animals with simulated chronic alcohol intoxication leads to improvement of the microstructure in cardiomyocytes compared with control rats. This indicates pronounced cardioprotective effects of the studied neuroactive amino acid derivatives.

Keywords: chronic alcohol intoxication, cardioprotective effect, GABA and glutamic acid derivatives

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Морфологические изменения миокарда крыс после хронической алкогольной интоксикации на фоне лечения новыми производными ГАМК и глутаминовой кислоты

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РЕЗЮМЕ

Цель – изучение патоморфологических изменений миокарда крыс после хронической алкогольной интоксикации (ХАИ) на фоне лечения новыми производными глутаминовой кислоты – глуфиметом (соединение РГПУ-238), гамма-аминомасляной кислоты (ГАМК) – соединением РГПУ-260.

Материалы и методы. Эксперименты проведены на самках крыс линии Wistar, в возрасте 10 мес, разделенных на группы: 1 – интактные самки; 2 – контрольная группа, животные после ХАИ, которая моделировалась заменой питьевой воды на 10%-й раствор этанола в течение 24 нед; 3 и 4 – экспериментальные группы, в которых самкам вводили, соответственно, глуфимет в дозе 28,7 мг/кг и РГПУ-260 в дозе 25 мг/кг внутрибрюшинно, однократно в течение 14 сут после прекращения алкоголизации. Животные группы 5 получали препарат сравнения милдронат в дозе 50 мг/кг в аналогичном с исследуемыми соединениями режиме. Оценивали изменение микроструктурных и морфометрических параметров миокарда левого желудочка с использованием световой микроскопии.

Результаты. У животных после ХАИ выявлены уменьшение объемной доли кардиомиоцитов с увеличением таковой интерстиция и сосудов, а также деструктивные изменения кардиомиоцитов в виде их волнообразной деформации, потери поперечной исчерченности, очагов плазмолиза и фрагментации мышечных волокон. У крыс с терапией глуфиметом после ХАИ структурные изменения мышечных клеток были минимальны, сопровождались незначительным отеком, сосуды менее полнокровны с единичными стазами и сладжами, объемная доля кардиомиоцитов была на 9,7% выше, а интерстиция и сосудов – на 66,0 и 70,0% соответственно ниже. У животных, получавших соединение РГПУ-260 после алкоголизации, отсутствовали выраженные дегенеративные изменения кардиомиоцитов и нарушения микроциркуляции в миокарде аналогично самкам, которым вводили глуфимет. Милдронат оказывал менее выраженное кардиопротекторное действие.

Заключение. Введение животным с моделированной хронической алкогольной интоксикацией новых производных ГАМК и глутаминовой кислоты вызвало улучшение микроструктурных и морфометрических характеристик миокарда по сравнению с контрольными крысами, что свидетельствует о выраженных кардиопротекторных эффектах изучаемых производных нейроактивных аминокислот.

Ключевые слова: хроническая алкогольная интоксикация, кардиопротекторное действие, производные ГАМК и глутаминовой кислоты

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Исследование одобрено Региональным исследовательским этическим комитетом Волгоградской области (протокол № 2034-2017 от 15.09.2017).

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INTRODUCTION

Diseases associated with alcohol consumption remain some of the most studied pathological conditions due to their high social significance. According to the World Health Organization (WHO), alcohol consumption is a leading risk factor for premature death and disability among middle-aged and young people [1]. Recently, an increase in the consumption of ethyl alcohol has been recorded, especially during the COVID-19 pandemic [2].

The consequences of alcohol consumption are associated with cardiovascular and neuropsychiatric diseases, cancer, as well as with liver, kidney, and endocrine diseases [3]. According to numerous studies, ethanol exerts one of the most damaging effects on the heart. Alcoholic cardiomyopathy (ACM) is the most prevalent form of ethanol-induced heart damage in chronic alcohol intoxication (CAI) [4].

Ethanol has negative effects on cardiomyocytes (CMs) damaging their membrane, receptors, mitochondria, ribosomes, cytoskeleton, and DNA. This is due to the small size of the ethanol molecule, its high reactivity and large volume of distribution in the body. Ethanol causes impairment of the plasma membrane, activation of lipid peroxidation and apoptosis, and disruption of signaling mechanisms [5]. Ethanol affects the structure of the myocyte cytoskeleton, connexons, and desmosomes, which causes structural instability of cells [6].

As a result, swelling and destruction of mitochondria lead to energy deficiency in CMs. These processes are accompanied by disorders of lipid metabolism and fatty degeneration of the heart. Ion exchange disorders cause fragmentation of myofibrils. Hypoxia, energy deficiency, electrolyte imbalance, and oxidative stress lead to severe atrophy and death of CMs and compensatory replacement of myofibrils with connective tissue [7]. A decrease in the volume fraction of CMs and changes in their microstructure as well as disorders of the excitation – contraction coupling and contractile protein synthesis cause a decline in myocardial contractility and development of heart failure (HF).

Currently, the principles of treatment for ACM mainly include metabolic therapy (meldonium, mexidol, etc.) and are directed to compensation for already developed HF. However, there is still no pathogen-specific correction of morphological and functional disorders. In this regard, search for pharmacological agents which exert cardioprotective effect in CAI remains relevant.

New derivatives of glutamic acid and gamma-aminobutyric acid (GABA) can be considered as such agents. Previous studies have shown cardioprotective effects of the glutamic acid derivative glufimet (dimethyl ester of 3-phenyl glutamic acid hydrochloride, RSPU-238 compound, Fig. 1, *a*) and the GABA derivative compound RSPU-260 (a two-component composition of methyl-4-amino-3- phenylbutanoate (mefebut) and L-arginine hydrochloride in the ratio of 1:1, Fig. 1, *b*) in CAI. Recently, glufimet and RSPU-260 have demonstrated their membrane-protective and antihypoxic effects. Application of these compounds contributes to an increase in inotropic myocardial reserve, improves endothelium-dependent vasodilation, and reduces lipid peroxidation [8, 9].

In this regard, the aim of the study was to assess the alcohol-induced pathohistological changes in the rat myocardium after treatment with glufimet and RSPU-260.

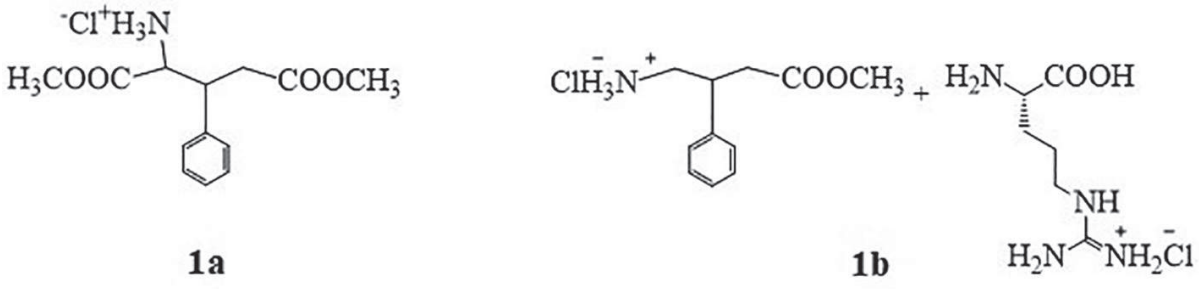

Fig. 1. Structural formulae of*: a*– glufimet, *b* – the compound RSPU-260

MATERIALS AND METHODS

Experiments were carried out on female Wistar white rats, aged 10 months and weighing 280–320 g. The rats were delivered from the Stolbovaya animal resource center (Russia, Moscow region). The animals were housed and maintained under the standard vivarium conditions with free access to food and water in 12 : 12 light / dark cycle. The study was

carried out in compliance with the Good Laboratory Practice (GLP) requirements for preclinical trials in the Russian Federation (Ministry of Health of the Russian Federation, order no. 199n of April 1, 2016 "On the Adoption of Rules of Good Laboratory Practice"), the International Recommendations of the European Convention for the Protection of Vertebrate Animals Used in Experiments or for Other Scientific Purposes (1986), and the European Union Directive 2010/63/EU 22.09.2010 on the protection of animals used for scientific purposes.

To simulate CAI, the rats were provided with a 10% (v/v) ethanol solution (RFK, Russia) sweetened with sucrose (50 g / l) as the only source of drinking for 24 weeks [10].

The rats were divided into the following groups: group 1 – intact females (n = 7); group 2 – control group, females after CAI (n = 7) receiving 0.1 ml saline solution per 100 g of weight after discontinuation of alcohol consumption; groups 3 and 4 – two experimental groups in which the female rats after CAI were administered with glufimet (n = 7) at a dose of 28.7 mg / kg and RSPU-260 (n = 7) at a dose of 25 mg / kg; group 5 – a group of animals receiving the reference listed drug mildronate (Grindex, Latvia) at a dose of 50 mg / kg (n = 7). Glufimet and RSPU-260 were synthesized at the Department of Organic Chemistry, Herzen Russian State Pedagogical University, St. Petersburg, Russia. Saline solution, the studied compounds (dissolved in saline), and the reference listed drug were injected intraperitoneally once a day for 14 days, starting from the day following CAI cessation.

The hearts were obtained from the lethally narcotized animals (chloral hydrate, 400 mg / kg). The muscle tissue blocks (0.5 x 0.8 cm) obtained from the left ventricle were fixed in 10% neutral buffered formalin for 24 hours. After being washed in running tap water for 6 hours, the tissue blocks were dehydrated and subsequently treated with xylene and placed in the HISTOMIX medium (Biovitrum, Russian Federation). Sectioning of the myocardial blocks was performed with the rotatory microtome HM340E (Thermo Fisher, USA), followed by mounting of the 5–6-μm slices on Polysine microscope adhesion slides (Thermo Scientific, USA). Subsequent to deparaffinization and rehydration, the myocardial sections attached to the microscope slides were stained with hematoxylin (NPF Abris+, Russia) and 0.5% alcoholic solution of eosin (Labiko LLC, Russian Federation). The stained sections on coverslips were mounted in the VitroGel medium (ErgoProduction LLC, Russian Federation) [11].

The morphometric examination involved the assessment of the digitized microphotographs of the rat myocardium processed with MCview software (LOMO-microsystems, Russian Federation). For the quantitative assessment of changes in the sections, we determined the volume fraction of CMs, vessels, and interstitium, thickness of CM wall, CM nuclear area in the longitudinal section, and CM cross-sectional area. To meet the data representativeness criterion, the morphometric measurements were taken in 10 randomly chosen fields of view for each section. The qualitative analysis of microstructural changes included the assessment of the following pathohistological features: the presence of focal and perivascular sclerotic lesions, atrophy (hypertrophy) of individual muscle fibers or their groups, wave-like deformation of CMs, foci of CM disintegration, foci of interstitial accumulation of lymphoid and lymphohistiocytic infiltrates, microhemorrhages, stasis and sludge in arterioles and venules, uneven coloration of CMs and their nuclei. We used the semi-quantitative method with a 1–4-point scale to assess the pathohistological changes [12].

The statistical analysis was performed using the Statistica 12.5 software. The Shapiro – Wilk test was used to evaluate the normality of data distribution. The Student – Newman – Keuls test was applied for pairwise comparison. The quantitative variables were presented as $M \pm SD$, where M is the mean, and SD is the standard deviation. The differences were considered statistically significant at $p < 0.05$.

RESULTS

The microscopic examination of the intact rats revealed no pathological changes in the myocardium. The cross-striated sarcoplasm of muscle fibers was well defined, the nuclei with weak polymorphism were located in the central compartment of CMs which were surrounded by a thin layer of loose connective tissue with single erythrostasis (Fig. 2, *a*).

CAI resulted in degenerative changes in the rat myocardium, such as perivascular and intermuscular edema with moderate hypertrophy and wave-like deformation of CMs, loss of cross-striation, foci of plasmolysis, and fragmentation of muscle fibers. Round and oval nuclei were hyperchromic (Fig. 2, *b*). Disturbances of microcirculation were manifested by plethora of the vessels, aggregation of erythrocytes, and petechial hemorrhages. Pronounced leukocyte

infiltration was observed in the muscle tissue. The morphometric analysis revealed an increase in the CM cross-sectional area by 9.2% ($p < 0.05$), a significant decrease in the CM volume fraction, and an increase in the volume fractions of the interstitium (by 98.0%, $p < 0.05$) and vessels (by 11.1%) in the female rats with CAI compared with the intact animals (Table).

The animals receiving glufimet showed less pronounced changes (Fig. 2, *c*). Cross-striation of muscle fibers was preserved, no edema and wave-like deformation were noted in CMs separated by thin layers of loose connective tissue. We also found less plethoric vessels and insignificant phenomena of stasis and sludge of erythrocytes in the microvasculature. The mean cross-sectional area of left ventricular CMs in this group of animals was close to the value in the intact rats and by 9.5% smaller than in the control rats ($p < 0.05$) (Table). It is worth noting that treatment with glufimet after CAI resulted in the greater volume fraction of CMs (by 9.7%, $p < 0.05$) and reduced volume fraction of the interstitium and vessels (by 66.0 and 70.0%, respectively, $p < 0.05$). In addition, the mean nuclear area in CMs significantly increased by 26.2% ($p < 0.05$) compared with the female rats after CAI.

The compound RSPU-260 had pronounced cardioprotective effects. After treatment with RSPU-260, minimal pathological changes in CMs and microcirculation, such as minor petechial hemorrhages and stasis of erythrocytes, were noted (Fig. 2, *d*). The interstitial volume fraction was by 42.3% ($p < 0.05$) smaller, and the CM volume fraction was significantly increased compared with the control group.

In the animals treated with the reference listed drug mildronate, the pathomorphological changes were more pronounced than in the groups which were injected with the experimental compounds (Fig. 2, *e*). The microscopic examination revealed hypotrophy of muscle cells, which was confirmed by the morphometric analysis data – the mean cross-sectional area of CMs was by 17.8% smaller than in the rats after CAI and by 10.2% smaller than in the intact animals. In the microvasculature, stasis and sludge of erythrocytes and minor petechial hemorrhages were noted. However, the CM volume fraction was significantly greater and the interstitial volume fraction was significantly smaller than in the controls ($p < 0.05$) (Table).

Table

Changes in the morphometric parameters of the left ventricular myocardium in the experimental rats after CAI in the context of treatment with the new GABA and glutamic acid derivatives, *M* ± *SD*

Animal groups	CM cross-sectional area, μm2	CM nuclear area, μm2	CM thicknesses, μm	CM volume fraction, %	Vascular volume fraction, %	Interstitial volume fraction, %
Intact group	241.4 ± 11.2	32.1 ± 2.7	12.8 ± 1.6	92.4 ± 1.4	2.7 ± 1.1	4.9 ± 0.8
CAI + saline	263.6 ± 14.3 *	33.6 ± 1.6	12.5 ± 1.3	87.3 ± 1.2 *	3.0 ± 0.9	9.7 ± 1.1 *
CAI + glufimet	238.5 ± 9.2 #	42.4 ± 4.6 #	12.7 ± 0.9	95.8 ± 0.9 #	0.9 ± 0.5 #	3.3 ± 0.7 #
CAI + RSPU-260	245.3 ± 13.3 #	30.7 ± 5.0	11.6 ± 0.7	92.2 ± 1.4 #	2.2 ± 0.9	5.6 ± 1.1 #
CAI + mildronate	216.7 ± 13.6 #	28.7 ± 2.3 #	11.1 ± 1.2 #	92.5 ± 2.7 #	2.4 ± 0.6	5.1 ± 1.1 #

* $p < 0.05$ relative to the group of intact animals (Student's *t* test);
# $p < 0.05$ relative to the control group of animals with CAI receiving saline (Newman – Keuls test).

DISCUSSION

Replacing drinking water with 10% ethanol solution for 24 weeks caused pathohistological changes in the left ventricular myocardium of the experimental rats. The morphometric analysis demonstrated a decrease in the CM volume fraction, which indicates a decrease in the number of functioning muscle cells and a rise in the interstitial volume fraction due to proliferation of fibroblasts and perivascular and intermuscular fluid accumulation. In the control group of animals after CAI, destructive changes in the CMs were revealed: they lost their cross-striation and had foci of plasmolysis and fragmentation of muscle fibers. Ethanol has damaging effects by directly affecting cellular structures and via production of reactive oxygen species and disruption of lipid metabolism and intracellular calcium homeostasis [13].

Ethyl alcohol impairs cell membrane integrity, increasing mobility and permeability of the phospholipid bilayer. These changes lead to disruption of membrane-associated proteins, resulting in disruption of their transport and signaling. Ethanol easily diffuses through the cell membrane and affects organelles in CMs, especially mitochondria. Ethyl alcohol reduces the membrane potential and activity of membrane respiratory chain complexes, which also damages mtDNA.

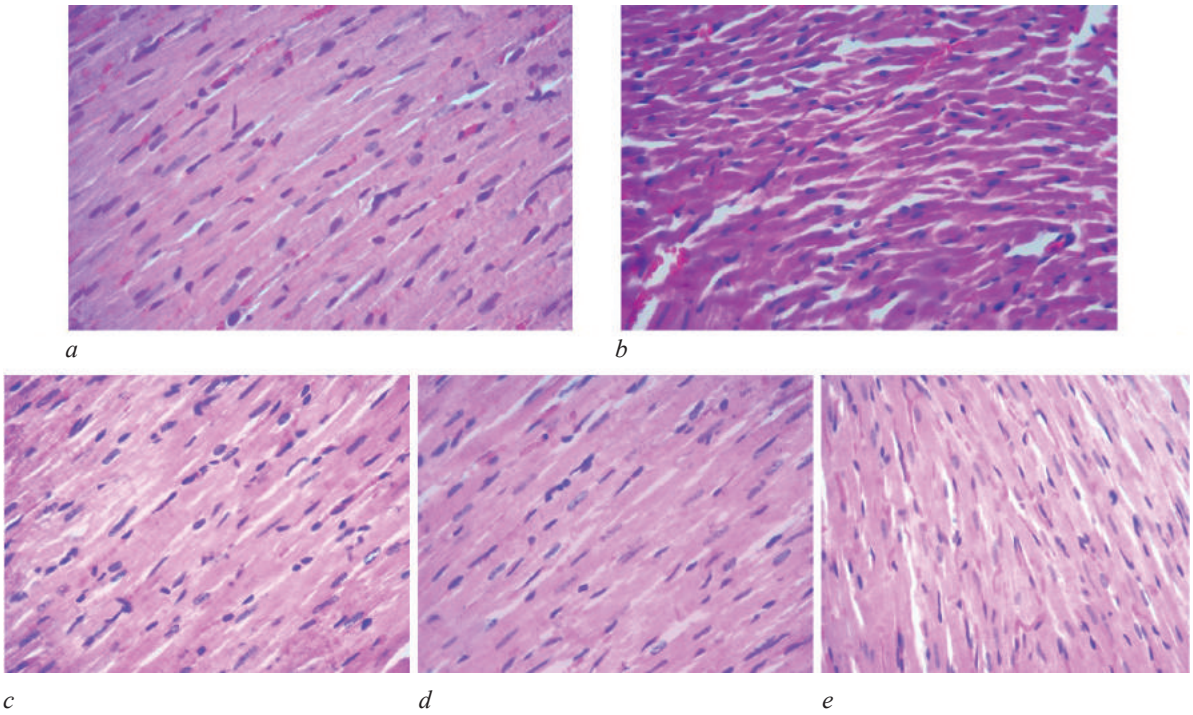

Fig. 2. Microscopic images of the left ventricular myocardium: *a* – in the intact rats, *b* – in the control group, *c* – after treatment with glufimet, *d* – after treatment with RSPU-260, *e* – after treatment with mildronate. Staining with hematoxylin and eosin, x400.

The described changes contribute to the development of hypoxia and ATP deficiency in alcohol-damaged cells. In support of this, the presented study showed a trend toward an increase in the vascular volume fraction which can be explained by stimulated angiogenesis under hypoxic conditions. Also, an increase in the cross-sectional area of CMs was revealed, indicating compensatory myocardial hypertrophy. In addition, mitochondrial dysfunction leads to active production of reactive oxygen species (ROS) involved in apoptosis of CMs [14], causing fragmentation of contractile proteins and dysfunction of the sarcoplasmic reticulum [15, 16].

In CAI, synthesis of CM structural proteins changes and the excitation – contraction coupling reduces [17]. Chronic alcohol consumption enhances expression of type I and III collagen in the myocardium, leading to fibrosis of the cardiac muscle tissue [18]. In addition, ethanol induces Ca^{2+} leakage from the sarcoplasmic reticulum and decreases sensitivity of myofilaments to calcium [19, 20]. The described processes exacerbate hypoxia and induce the development of fatty infiltration in the myocardium, fragmentation of myofibrils, compensatory CM hypertrophy, as well as necrosis and apoptosis with the compensatory growth of connective tissue. As a result, the contractile function of the myocardium decreases and chronic HF develops leading to severe disability and death.

The new glutamic acid derivative glufimet limited the negative effects of ethanol on the myocardium. In the rats treated with glufimet, degenerative changes in CMs were minimal; they were accompanied by minor edema. The microvasculature was less plethoric with insignificant phenomena of stasis and sludge. The mean cross-sectional area of CMs treated with glufimet was significantly smaller than that in the control rats with CAI and similar to that in the intact animals. Cardioprotective effects of glufimet are probably associated with incorporation of glutamate and glycine fragments in its chemical formula.

Glutamic acid has a wide range of metabolic effects. This amino acid can improve myocardial tolerance to hypoxia due to intensification of anaerobic glycolysis in the cytosol and regeneration of NAD^+, participation in the malate – aspartate shuttle, and activation of the electron transport chain in mitochondria. In addition, glutamate probably contributes to restoration of oxidative metabolism due to replenishment of Krebs cycle intermediates, for example, α-ketoglutarate [21]. It is known that glycine is involved in detoxification reactions and reduces the intensity of lipid peroxidation, being a part of glutathione, a tripeptide with pronounced

antioxidant activity. It also has cytoprotective and anti-inflammatory effects [22].

The new GABA derivative RSPU-260 was as effective as glufimet. In the animals treated with RSPU-260 after 24-week alcohol consumption, no pronounced degenerative changes in CMs and disorders of myocardial microcirculation were revealed. GABA, like glutamic acid, has metabolic effects, being a precursor of succinate, a Krebs cycle intermediate. Therefore, an increase in succinate, the substrate of complex II of the electron transport chain, stimulates ATP synthase and formation of ATP in the cell [23]. In addition, GABA limits excessive sympathetic influences on the heart, supporting functional reserves of the myocardium. The RSPU-260 compound contains L-arginine, a substrate for synthesis of nitric oxide, a bioactive molecule with a number of cardio- and endothelium-protective effects.

Mildronate had a less pronounced cardioprotective effect. In the rats treated with the reference listed drug, muscle fiber atrophy and minor microcirculation disorders were observed. Mildronate regulates energy metabolism by reducing synthesis and biological activity of L-carnitine, stimulating glucose metabolism [24].

CONCLUSION

The new GABA and glutamic acid derivatives stabilized the microstructural and morphometric parameters of the myocardium after CAI in the experimental animals. The results can be used for further research and development of new drugs to optimize pharmacotherapy of heart diseases associated with CAI.

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Artificial neural networks in predicting impaired bone metabolism in diabetes mellitus

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ABSTRACT

Growing incidence of diabetes mellitus (DM), given significant socioeconomic consequences that low-trauma fractures entail, determines a need to improve diagnostic standards and minimize the risk of medical errors, which will reduce costs and contribute to better treatment outcomes in this category of patients.

Aim. To assess diagnostic capabilities of the method based on the use of an artificial neural network (ANN) for predicting changes in reparative osteogenesis in diabetes mellitus.

Materials and methods. A single-center, one-stage, cross-sectional study included 235 patients with type 1 and type 2 diabetes mellitus and 82 persons of the control group (the total of 317 patients). Further, the obtained data were processed using the MATLAB software to develop an ANN with a training (80%) and test (20%) set. The ANN model was trained by optimizing the relationship between a set of input data (a number of clinical and laboratory parameters: gender, age, body mass index, duration of diabetes mellitus, etc.) and a set of corresponding output data (variables reflecting the state of bone metabolism: bone mineral density, markers of bone remodeling).

Results. The ANN-based algorithm predicted estimated values of bone metabolism parameters in the examined individuals by generating output data using deep learning. Machine learning was repeated until the error was minimized for all variables. The accuracy of the validation test to predict changes in bone metabolism based on patient data was 92.86%.

Conclusion. The developed ANN-based method made it possible to design an auxiliary tool for stratification of patients with changes in bone metabolism in diabetes mellitus, which will help reduce healthcare costs, speed up the diagnosis due to fast data processing, and customize treatment for this category of patients.

Keywords: diabetes mellitus, osteopathy, neural networks

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Azerbaijan Medical University (Protocol No. 02/14 of 12.10.2016).

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Искусственные нейронные сети в прогнозировании нарушений метаболизма костной ткани при сахарном диабете

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РЕЗЮМЕ

По мере роста заболеваемости сахарным диабетом, и учитывая существенные социально-экономические последствия, которые влекут за собой низко травматические переломы, возникает необходимость в коррекции стандартов диагностики и сведении к минимуму риска медицинских ошибок, что позволит снизить затраты и добиться лучших результатов в лечении данной категории больных.

Цель: оценка диагностических возможностей метода, основанного на применении искусственной нейронной сети (ИНС) в качестве инструмента прогнозирования изменений процессов репаративного остеогенеза при сахарном диабете.

Материалы и методы. Выборка была сформирована в ходе исследования 235 пациентов с сахарным диабетом 1-го и 2-го типа и 82 лиц контрольной группы (всего 317 человек). Далее набор полученных данных был обработан программным обеспечением MATLAB для построения ИНС с обучающим (80%) и тестовым (20%) набором. Модель ИНС обучалась путем оптимизации взаимосвязи между набором входных данных (показатели: пол, возраст, индекс массы тела, длительность диабета и т.д.) с набором соответствующих выходных данных (переменных, отражающих состояние костного метаболизма: минеральную плотность кости, маркеры костного ремоделирования).

Результаты. Базируемый на ИНС алгоритм с высокой точностью способен спрогнозировать значения показателей метаболизма костной ткани обследованных пациентов, сгенерировав выходные данные с помощью глубокого обучения. Процесс машинного обучения повторялся до тех пор, пока не минимизировалась ошибка для всех переменных. Точность валидационного теста для прогнозирования изменения костного метаболизма на основе данных пациентов составила 92,86%.

Заключение. Применение аппарата искусственных нейронных сетей позволило сконструировать вспомогательный инструмент для стратификации пациентов с сахарным диабетом, имеющих нарушения репаративного остеогенеза, что может помочь сократить затраты на обследование, ускорить диагностику за счет быстрого процесса обработки данных и скорректировать процесс лечения данной категории пациентов.

Ключевые слова: сахарный диабет, остеопатия, нейронные сети

Конфликт интересов. Автор декларирует отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источники финансирования. Автор заявляет об отсутствии финансирования исследования при проведении исследования.

Соответствие принципам этики. Все пациенты подписали информационное согласие на участие в клиническом исследовании. Исследование одобрено локальным этическим комитетом Азербайджанского медицинского университета (протокол № 02/14 от 12.10.2016).

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INTRODUCTION

Growing incidence of diabetes mellitus (DM), given significant socioeconomic consequences that low-trauma fractures entail, determines a need to improve diagnostic standards and minimize the risk of medical errors, which will reduce costs and contribute to better treatment outcomes in this category of patients. Metadata indicate higher incidence of bone fractures in patients with type 1 and type 2 DM compared with the general population [1–3]. Methods commonly used to diagnose bone diseases in DM include

analysis of hormonal and electrolyte homeostasis, bone remodeling markers, and dual-energy X-ray absorptiometry (DXA). All these methods have certain limitations. In addition, diagnosis is complicated by the peculiarities of the pathogenesis of DM and is aggravated by impaired fracture healing [4].

Traditionally, the use of diagnostic methods can be minimized by data mining techniques to identify and analyze hidden information within data to better predict results and accelerate and personalize the diagnosis of bone changes in DM. These techniques include artificial intelligence, statistics, machine learning, and visualization [5].

Decision support systems (DSS) based on artificial neural networks (ANN) are effectively used in clinical diagnostics, which is why these tools are becoming more and more popular for developing individualized diagnostic approaches and predicting a number of diseases, and, as a result, developing an optimal treatment strategy due to a more accurate and rapid analysis of interrelated processes in the body [1, 5]. Nevertheless, ANN has never been used in predicting the risk of bone fractures in DM based on routine clinical and laboratory tests.

The **aim** of the study was to assess the diagnostic capabilities of the method based on the use of ANN as a tool for predicting changes in reparative osteogenesis in DM.

MATERIALS AND METHODS

A single-center, one-stage, cross-sectional study included 235 patients with type 1 and type 2 DM and 82 persons of the control group (the total of 317 patients).The study was conducted in 2015–2017 at the Department of Endocrinology of the Educational and Therapeutic Clinic at Azerbaijan Medical University. All parameters were evaluated once.

The exclusion criteria were: bone fractures in the past medical history, treatment for osteoporosis in the medical history, endocrine diseases, non-diabetic liver and kidney diseases, and final stages of diabetic nephropathy (stage IV–V).

For all patients, we indicated sex, age, duration of DM, body mass index (BMI) and measured bone mineral density (BMD), which was defined by T- and Z-scores in the lumbar spine (T–SD L1–4; Z–SD L1–4) and femoral neck (T–SD FN; Z–SD FN) by DXA. The levels of glycated hemoglobin (HbA1c), serum bone remodeling markers (alkaline phosphatase (ALP), procollagen type I N-terminal propeptide (PINP), and beta-C-terminal telopeptide (b-CTx)), parathyroid hormone (PTH), calcitonin (CT), vitamin D3 (25 (OH) D), and insulin were determined. We measured HOMA-IR index, glomerular filtration rate (GFR), and albumin. The ionic balance in the blood was also determined: total calcium (tCa) and ionized calcium (Ca^{2+}), phosphorus (P^{+}), magnesium (Mg^{2+}), potassium (K^{+}), and sodium (Na^{+}).

Statistical analysis. Following the previous clinical and laboratory research, we developed an ANN-based method to predict and assess the state of bone tissue in DM. The data were collected from individuals with type 1 and type 2 DM and processed by variational mathematical methods of statistical analysis using BioStatPro 6.2.2.0 software. Next, the data set was processed by MATLAB software (Attaway, 2022). The train – test split was 80% / 20%, respectively, with subsequent validation and normalization. The ANN model was trained by optimizing the relationship between a set of input data (a number of clinical and laboratory parameters: sex, age, BMI, duration of DM, etc.) and a set of corresponding output data (variables reflecting the state of bone metabolism: BMD, markers of bone remodeling). The accuracy of the validation test for predicting changes in bone metabolism in relation to the training data was 92.86%.

Data set development. The study was single-center, transverse and included the results of the examination of 98 patients with type 1 DM and 137 patients with type 2 DM. The control group included 82 individuals. The characteristics of all 317 patients are shown in Table.

Table

Clinical characteristics of patients, *M* (95% CI)

Parameter	Type 1 DM, *n* = 98	Type 2 DM, n = 137	Controls, n = 82
Age, years	55.8 (54.4–57.3)	58.4 (57.3–59.5)	55.9 (54.2–57.7)
Sex male / female	41/57	52/85	39/43
BMI, kg / m^2	26.1 (25.6–26.5)	30.0 (29.4–30.6)	28.7 (27.9–29.5)
DM duration, years	16.6 (15.4–17.8)	8.1 (7.2–8.8)	–
HbA1c, %	7.4 (7.1–7.8)	7.5 (7.2–7.8)	4.9 (4.7–5.0)
C-peptide, ng / ml	–	4.3 (1.64–7.4)	3.7 (3.1–4.7)

Table (continued)

Parameter	Type 1 DM, *n* = 98	Type 2 DM, n = 137	Controls, n = 82
HOMA-IR	–	8.6 (7.5–9.6)	2.8 (2.4–3.1)
tCa, mg / dl	9.3 (9.1–9.5)	9.4 (9.3–9.5)	9.5 (9.4–9.7)
Ca^{2+}, mmol / l	1.09 (1.07–1.11)	1.07 (1.04–1.08)	1.13 (1.11–1.15)
P^{+}, mg / dl	5.4 (5.2–5.6)	5.0 (4.8–5.2)	5.1 (4.9–5.2)
Mg^{2+}, mg / dl	1.52 (0.69–2.45)	1.54 (1.45–1.63)	1.75 (1.61–1.89)
K^{+}, mg / dl	4.4 (3.1–5.9)	4.3(4.1–4.4)	4.3 (4.1–4.5)
Na^{+}, mmol / l	142.2 (140.6–143.8)	140.9 (139.6–142.3)	138.5 (137.2–39.6)
Creatinine, mg / dl	0.85 (0.81–0.89)	0.79 (0.76–0.82)	0.75 (0.72–0.78)
GFR, ml / min / 1.73 m^2	86.7 (83.1–90.4)	88.5 (85.4–91.5)	95.2 (91.8–98.6)
Albumin, g / dl	4.2 (4.1–4.3)	4.3 (4.1–4.4)	4.5 (4.3–4.6)
PTH, ng / dl	51.16 (47.17–55.13)	51.69 (48.82–54.56)	45.09 (40.38–49.79)
Vitamin D_3,ng / ml	24.09 (21.32–26.86)	25.12 (22.98–27.28)	30.41 (26.95–33.86)
CT, pg / ml	12.07 (9.75–14.38)	10.23 (8.84–11.62)	5.5 (4.19–6.84)
ALP, UI / l	118.3 (110.1–126.4)	122.2 (116.2–128.1)	123.5 (113.8–133.2)
P1NP, ng / ml	40.58 (37.18–43.98)	42.08 (39.81–44.35)	47.09 (42.82–51.35)
b-CTX, ng / ml	0.525 (0.468–0.582)	0.495 (0.456–0.533)	0.424 (0.383–0.466)
T-score (T–SD L1–L4)	–1.4 (–2.2–(–0.9))	–1.08 (–1.3; –0.8)	–0.73 (–1.1; –0.3)
T-score (T–SD FN)	–1.15 (–1.9–(–0.7))	–1.12 (–1.3; –0.8)	–0.64 (–1.0; –0.2)

In this study, we modeled ANN which included the following input data: sex, age, weight, height, BMI, duration of DM, duration of menopause, blood glucose, HbA1c, albumin, creatinine, insulin, C-peptide, HOMA-IR index, tCa, Ca^{2+}, P^{+}, Mg^{2+}, K^{+}, Na^{+}, GFR, PTH, CT, vitamin D_3. Output data included the following Parameters: ALP, P1NP, b-CTX, T–SD L1–4, Z–SD L1–4. The input value was converted to the output value according to the activated functions shown in Fig. 1.

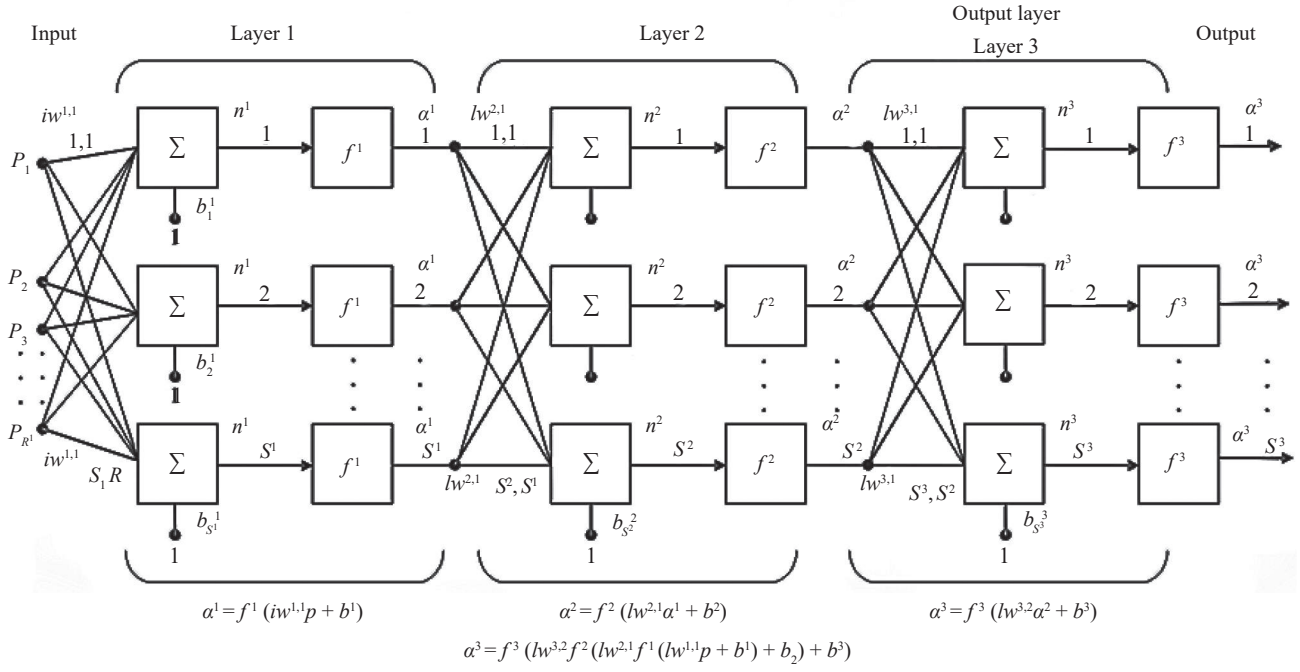

Fig. 1. Multilayer neural network

The proposed method for constructing a ANN-based self-learning predictive system for intelligent DSS that diagnoses clinical and pathogenic features of reparative osteogenesis in DM encompasses the following steps: setting a problem, preparing input data, dividing data into training and test sets, building and training a neural network including a multilayer perceptron (MLP) model, forming a training sample

supply scheme that determines a set of input elements and corresponding output neurons, additional training, and, if necessary, redesigning based on an assessment of the diagnosis accuracy. The elements of the input signals were normalized (in the range from –1 to 1); the input synapse was supplied not with the parameter value, but with its equivalent, recalculated by the formula:

$$NX_i(t) = \frac{X_i(t) - X_i^{\min}}{X_i^{\max} - X_i^{\min}},$$

where $X_i(t)$ is the original signal, $NX_i(t)$ is the received normalized signal, $X_i^{\min}$ and $X_i^{\max}$ are the minimum and maximum values for the input parameter intervals in the field supplied to synapse i.

The Bayesian Regularization algorithm (trainer) was used to train the ANN. The backpropagation learning algorithm was based on gradient descent search algorithms that allow the correlation weight to be modified to optimize the model. The test set was used for validation grading at the end of each training stage and testing at the end of training to get an idea of how well the model coped with the problem. The initial training of the model was carried out using the data of laboratory and instrumental studies of 317 patients listed above (Table). After the implementation of the above stages, the ANN was ready for the last stage, which was predicting biomarkers of reparative osteogenesis and BMD, which are informative for early diagnosis of impaired bone remodeling in DM, i.e. parameters used to control diagnostic processes.

Practical implementation of a neural network model for assessing the risk of osteoporotic changes in bone tissue in diabetes mellitus. The ANN was constructed using MATLAB software (Attaway, 2022) [6], and a visual interface was developed using the GUIDE tool to make it convenient for clinicians (Fig. 2).

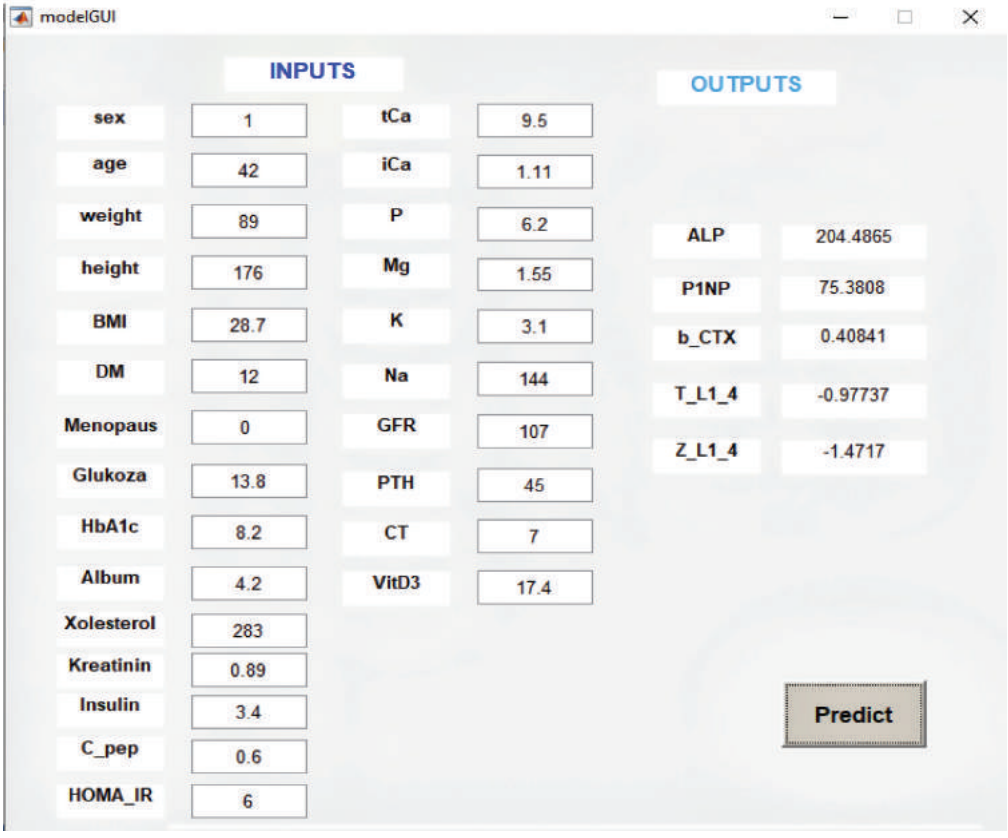

Fig. 2. Example of a calculation carried out by the ANN

RESULTS

In type 1 DM, both biochemical bone remodeling markers and DXA are informative indicators reflecting the state of bone metabolism. Biomarkers of bone remodeling can be of great importance in assessing the state of bone tissue, when the measurement of BMD is not informative enough, in particular, at the initial stages of type 2 DM (the data are given in Table). Due to the current situation, we applied the ANN to patient

data to determine the accuracy of predictions. The data from the examination of 317 patients were analyzed, of which 80% and 20% were used as training and test sets, respectively. An ANN-based DSS model was created to predict the state of bone metabolism in a particular patient with DM based on the input data: duration of DM, HbA1c values, vitamin D_3, etc. While developing DSS, some adjustments were made to the model settings to improve its adequacy. Further training was conducted during its practical operation. The learning process continued until the errors in all variables were minimized, and it was stopped at the moment when the error in the control sample began to increase. The accuracy of the validation test for predicting changes in bone metabolism in relation to the training data was 92.86%. The ANN-based algorithm predicted with high accuracy the values of the bone metabolism parameters in the examined individuals, generating output data via implementation of the hidden level (Fig. 3).

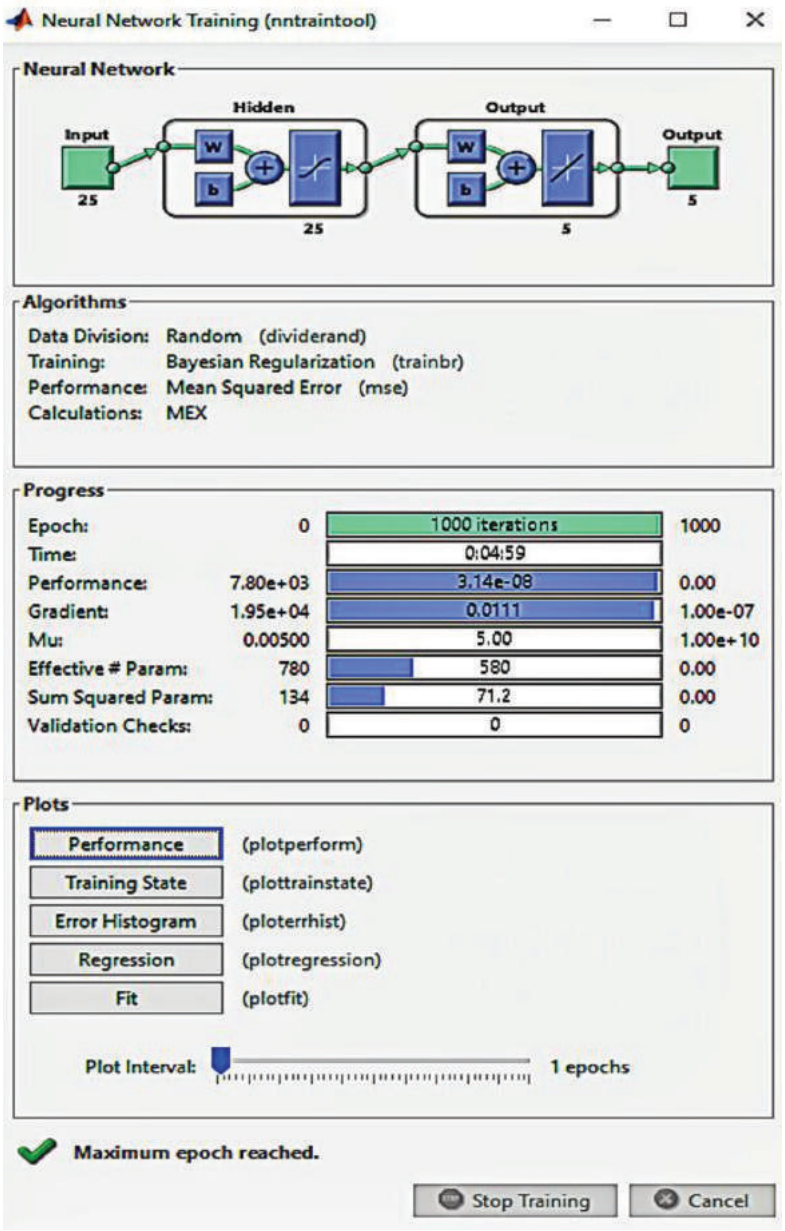

Fig. 3. ANN operation scheme

The results of the study showed the effectiveness of the developed method based on the construction of an intelligent DSS in identifying the relationships between bone metabolism parameters and a number of factors associated with DM.

DISCUSSION

Metabolic disorders caused by DM can negatively affect BMD, increasing the likelihood of low-trauma fractures which can lead to disability or death [1, 2]. Predicting a risk of fractures in DM patients is more challenging because the DXA method and the FRAX fracture risk calculator underestimate the risk of fractures in DM patients due to higher BMD [4]. Under these conditions, biomarkers of reparative osteogenesis can show the quality of bone tissue. However, high cost of these methods and low availability of appropriate equipment in the regions of Russia can complicate early diagnosis. A possible solution to this problem is introducing advanced computing technologies that can be integrated into the diagnosis and prediction of changes in reparative osteogenesis. At the same time, it should be kept in mind that in each individual case, the doctor deals with a pathological process with individual characteristics, both known and unknown [1]. The use of artificial intelligence methods reveals their wide possibilities and high efficiency in establishing links between data of clinical and instrumental studies and symptoms of the disease, which makes it possible to consider such systems as a practical tool for the doctor in the analysis and processing of complex clinical data.

The effectiveness of applying the ANN mathematical apparatus in clinical practice is justified by a wide range of its predicting capabilities based on processing of large, interconnected, multi-parametric arrays of medical data, which eliminates the need for expensive diagnosis and prevents improper treatment [1, 7]. Data classification and clustering algorithms, modeled in this study using ANN, showed efficiency and good capability to predict the presence or absence of low-trauma fracture risk in DM.

CONCLUSION

The developed method based on the mathematical apparatus of ANN made it possible to design an auxiliary tool for stratification of DM patients with changes in bone metabolism, which will help reduce healthcare expenses, speed up diagnosis through fast data processing, and adjust the treatment process for such patients.

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Subpopulations of B lymphocytes in patients with breast cancer depending on the PD-L1 status

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ABSTRACT

Aim. To study the association between the functional potency and degree of maturity of B lymphocytes and PD-L1 expression in breast cancer patients.

Materials and methods. The study included 37 patients with the morphologically verified diagnosis of invasive breast cancer of no special type (IBC NST). The PD-L1 status was determined immunohistochemically using the Ventana SP142 assay (Roche, USA). Using the multiplex flow cytometry-based assay and high-throughput sequencing of the tumor microenvironment, subpopulations of B lymphocytes and their CD27 and PD1 expression profiles were determined, taking into account the PD-L1 status.

Results. In the tumor microenvironment, regardless of the PD-L1 status, expression signatures of five lymphocyte subpopulations were determined. However, in PD-L1-positive patients, the levels of B lymphocytes and immunoglobulin class-switched B lymphocytes were higher compared with PD-L1-negative patients. Evaluation of the number of different B lymphocyte subpopulations by flow cytometry showed that PD-1-positive B lymphocytes predominated in the tumor microenvironment in PD-L1-positive patients, regardless of the degree of lymphocyte maturity.

Conclusion. The results of the study showed predominance of mature committed B lymphocytes and memory B lymphocytes capable of synthesizing immunoglobulins of different classes and Th2 cytokines involved in type 2 immune response in PD-L-positive tumor microenvironment. It suggests that immunotherapy with PD-L1 inhibitors is highly likely to activate cells with protumor potential and can ultimately contribute to breast cancer progression.

Keywords: breast cancer, tumor microenvironment, B lymphocytes, PD-L1

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Cancer Research Institute, Tomsk NRMC (Protocol No.7 of 25.08.2020).

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Субпопуляции В-лимфоцитов у больных раком молочной железы в зависимости от статуса PD-L1

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РЕЗЮМЕ

Цель. Изучить, насколько функциональные потенции и степень зрелости В-лимфоцитов сопряжены со статусом экспрессии PD-L1 опухоли у больных раком молочной железы.

Материалы и методы. В исследование вошли 37 пациентов с морфологически верифицированным диагнозом инвазивной карциномы молочной железы неспецифического типа (ИКНТ). Статус PD-L1 определялся иммуногистохимически тестом Ventana SP142 (Roche, США). С использованием методов мультиплексной проточной цитофлуориметрии и высокопроизводительного секвенирования микроокружения были определены субпопуляции В-лимфоцитов, их профиль экспрессии CD27 и PD1 с учетом статуса PD-L1 опухоли.

Результаты. В микроокружении опухоли у пациентов независимо от статуса PD-L1 определяются экспрессионные сигнатуры пяти субпопуляций лимфоцитов. Однако у больных с позитивным статусом в микроокружении первичной опухоли уровни В-лимфоцитов и В-лимфоцитов с переключаемым классом Ig выше по сравнению с пациентами, имеющими негативный статус PD-L1. Оценка количества различных субпопуляций В-лимфоцитов методом проточной цитофлуориметрии показала, что у больных с PD-L1-позитивным статусом опухоли в микроокружении преобладают PD-1-позитивные В-лимфоциты независимо от степени их зрелости.

Заключение. Результаты исследования показывают преобладание зрелых коммитированных В-лимфоцитов и В-лимфоцитов памяти, способных к синтезу иммуноглобулинов разных классов и цитокинов, относящихся к спектру иммуновоспалительных реакций Th2 типа в микроокружении PD-L1-позитивных опухолей. Это может являться неблагоприятным признаком при планировании иммунотерапии анти-PD-L1 ингибиторами, поскольку с высокой вероятностью ее применение может активировать клетки микроокружения с проопухолевыми потенциями, что в конечном итоге будет способствовать прогрессии карцином.

Ключевые слова: рак молочной железы, микроокружение, В-лимфоциты, PD-L1

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INTRODUCTION

In the last decade, doctors began to actively use a new class of immunotherapy drugs, immune checkpoint inhibitors (ICIs), which block the PD-1 / PD-L1-dependent mechanism of immune response suppression in the tumor. New options for medical oncologists were developed providing patients with many years to live. However, the analysis of the ratio of indications for prescription to the benefit from these drugs in a cohort of American patients clearly demonstrated an increase in the number of indications but no proper increase in effectiveness [1]. Moreover, the evaluation of the effectiveness of ICI

therapy indicates that a prolonged response is indeed observed only in some patients. The other group of patients have at best stabilization of the disease and at worst – progression and hyperprogression of pathology [2].

It is known that the type of the immune and inflammatory response in the breast cancer microenvironment can determine the response to therapy and the course and prognosis of the disease. However, to date, despite numerous studies, there are no acceptable predictors of the response to ICIs other than the assessment of PD-L1 expression and MMR deficiency [3].

The role of B lymphocytes in the tumor is quite controversial. It is well known that B lymphocytes contribute to tumor progression. In particular, this was shown in human ovarian cancer [4]. Being some of the cellular elements of type 2 immune and inflammatory responses, B lymphocytes are the source of corresponding cytokines, which reduce the antitumor response [5]. By synthesizing transforming growth factor (TGF-β) and interleukin 10 (IL-10), B lymphocytes can promote differentiation and recruitment of regulatory T lymphocytes, resulting in an increased immunosuppressive microenvironment in the tumor and stimulating breast cancer metastasis [6]. Antitumor antibodies synthesized by plasma cells, which are descendants of B lymphocytes, can activate macrophages and promote invasive tumor growth and angiogenesis [7].

However, current data suggest that B lymphocytes in the tumor are a favorable prognostic marker in breast cancer [8]. In addition, there are observations indicating antitumor effects of B lymphocyte subpopulation with the CD27 memory cell phenotype. The presence of these cells in the microenvironment along with CD8+ T lymphocytes is associated with a favorable prognosis in ovarian cancer [9]. It is known that the presence of B lymphocytes in tumor tertiary lymphoid structures is associated with a more favorable disease prognosis [10]. Regarding the role of PD1 expression on B lymphocytes, it is known to be more a sign of maturity and weaker ability to proliferate and switch antibody isotypes [11]. Thus, in the context of immunotherapy, the protumor or antitumor functional characteristics of B lymphocytes are of great importance. It is important to understand to what extent the functional potency and degree of maturity of B lymphocytes are correlated with the expression status of PD-L1 and the presence of the PD1 receptor.

MATERIALS AND METHODS

Patients. The study included 37 patients with a morphologically verified diagnosis of invasive breast carcinoma of no special type (IC NST). The average age of patients who were treated at Cancer Research Institute, Tomsk NRMC, with estrogen receptor positive (luminal A and B-1, -2) and triple-negative cancer subtypes ($T_{1–3}$ $N_{0–3}$ M_0) was 52.7 ± 9.3 years. The patients did not receive neoadjuvant chemotherapy prior to surgical treatment. Fresh primary tumor samples were obtained during surgery and were then stored at –80 °C. The study was carried out in accordance with ethical standards (the Declaration of Helsinki of the World Medical Association "Ethical principles for medical research involving human subjects" as amended in 2000 and "Rules of Good Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Healthcare of the Russian Federation No. 266 of 19.06.2003). All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Cancer Research Institute, Tomsk NRMC (Protocol No. 7 of 25.08.2020).

Assessment of tumor-infiltrating lymphocyte (TIL) density in a primary tumor. The density of TILs in the primary tumor was assessed according to the recommendations of the International TILs Working Group for the evaluation of tumor-infiltrating lymphocytes [12]. To do this, a representative tumor sample was stained with hematoxylin and eosin according to the standard operating procedure. Next, the proportion of stroma occupied by mononuclear leukocytes was determined (Axio Scope A1 (Zeiss, Germany)). Areas with artifacts, areas of necrosis, and pronounced hyalinosis were excluded from the assessment. The value was expressed as a percentage of the stromal area.

Determination of the PD-L1 status. PD-L1 expression was assessed using the PD-L1 test (SP142, Ventana) in the BenchMark ULTRA system (Roche, USA). Staining of immune cells (ICs) which are present in the intratumoral and adjacent peritumoral stroma was assessed. These cells included lymphocytes, macrophages, dendritic cells, and polymorphonuclear leukocytes. ICs were estimated as the fraction of the tumor area occupied by them with PD-L1 immunostaining of any intensity. Human tonsil tissue was used as a control. The test was considered positive at IC ≥ 1%.

Flow cytometry. Fresh frozen samples of the primary tumor were used as the study material.

To obtain a suspension of primary tumor cells, its fragment was placed in the Medicon (50 μm, BD Biosciences) and homogenized in 1 ml of phosphate-buffered saline (BD Biosciences) for 1 min twice. The suspension was then filtered through a cell separation mesh (70 μm, Falcon, Japan). The cells were then washed twice and resuspended in 100 μl cell staining buffer (Sony Biotechnology) and stained with a cocktail of monoclonal antibodies: BV570-anti-CD45 (clone HI30, mouse IgG1, Sony Biotechnology, Japan), PerCP/Cy5. 5-anti-CD3 (clone UCHT1, Mouse IgG1, Sony Biotechnology, Japan), APC/Cy7-anti-CD20 (clone 2H7, Mouse IgG2b, Sony Biotechnology, Japan), BV785-anti-CD27 (clone O323, Mouse IgG1, Sony Biotechnology, Japan), BV510-anti-CD28 (clone CD28. 2, Mouse IgG1, Sony Biotechnology, Japan), BV605-anti-CD279 (PD-1) (clone EH12.2H7, Mouse IgG1, Sony Biotechnology, Japan), BV421-anti-CD274 (PD-L1) (clone MIH3, Mouse IgG1, Sony Biotechnology, Japan), and AF647-anti-CD326 (EpCAM) (clone 9C4, Mouse IgG2b, Sony Biotechnology, Japan). Unstained and isotype controls were used. Isotype antibodies were added to the corresponding isotype control in a similar concentration. The analysis was performed on the NovoCyte 3000 flow cytometer (ACEA Biosciences, Agilent, USA). Cell populations were gated based on determination of forward scatter (FSC) and side scatter (SSC) parameters. Then the cells were analyzed for fluorescence in Density Plot and Dot Plot modes. The number of cells was presented as a fraction of all lymphocytes.

Sequencing of primary tumor cell microenvironment. Frozen tumor samples were taken from eight patients (PD-L1 negative – four patients, PD-L1 positive – four patients). Seven-micrometer-thick sections were prepared on PEN membrane frame slides (Carl Zeiss, Oberkochen, Germany) pre-treated with RNAZap (Thermo Fisher Scientific, Waltham, MA, USA) and stained with hematoxylin and eosin. PALM microbeam laser capture microdissection (Carl Zeiss, Oberkochen, Germany) was used to isolate cells from the tumor microenvironment. Four samples of stromal fragments adjacent to tumor cells were isolated from each tumor. A total of 32 samples were collected. RNA was isolated using the Single Cell RNA Purification Kit (Norgen, Canada). cDNA libraries were prepared using the SMARTer Stranded Total RNA-Seq kit v2 (Takara, USA). The size of cDNA libraries was estimated using the HS D1000 ScreenTape kit and 2200 Tape Station (Agilent, USA) and varied from 200 to 700 bp, with an average peak size of 340 bp.

The concentration of cDNA libraries was estimated using the Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA) and varied from 2.5 to 14 ng / μl depending on the number of cells in the microdissected samples. Samples of cDNA libraries were pooled, denatured, and sequenced on NextSeq 500 (Illumina, USA) in a single-end sequencing read mode for 75 cycles. The number of clusters was 220 K / mm^2 (93% of clusters after filtration), and the number of reads was ~12 million per sample. The bioinformatic analysis included mapping of reads using the STAR software (the GRCh38 reference genome assembly and GENCODE.R27 annotation). The number of reads in the coding and non-coding regions of the genome in each sample was estimated using the featureCounts tool. The xCell algorithm was used to identify cell types in the microenvironment [13]. The sequencing data were published in the GEO database (No. GSE184196).

Statistical analysis. Categorical parameters were compared using the Fisher's exact test. Differences in independent quantitative variables were assessed using the Mann–Whitney test. All criteria were two-sided and considered significant at $p < 0.05$. The analysis was performed using GraphPad Prizm 9 software.

RESULTS

Patient characteristics. A total of 54% (20/37) of all patients included in the study had PD-L1 positive status and 46% (17/37) had PD-L1 negative status. The clinical and pathological characteristics of the two groups of patients were comparable. Menopausal patients over 50 years of age, with a tumor size between 20 and 50 mm, stage IIA grade 2 luminal B subtype prevailed.

Expression signatures of B lymphocytes in the breast cancer microenvironment. xCell analysis detects signatures of five B lymphocyte subsets. Signatures of all five lymphocyte subpopulations were determined in the tumor microenvironment in patients regardless of the PD-L1 status (Fig. 1).

The numbers of B lymphocyte subpopulations were not equal within the study groups (Table). Thus, PD-L1 negative patients had lower levels of naive B lymphocytes compared to the levels of B lymphocytes and memory B lymphocytes. The levels of B lymphocytes differed more markedly in PD-L1 positive patients. Thus, B lymphocytes and Ig class-switched B lymphocytes were predominant.

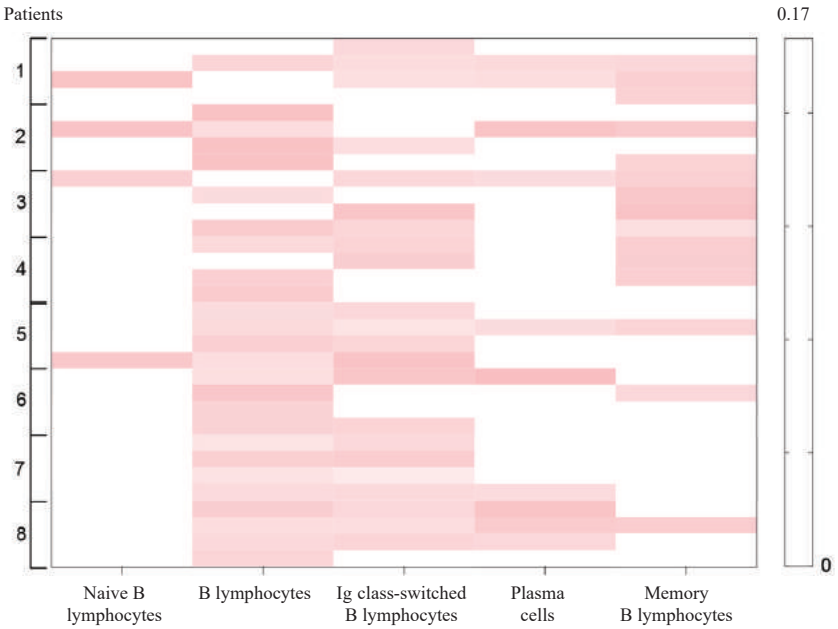

Fig. 1. Heat map of B lymphocyte signatures in the primary tumor microenvironment in breast cancer patients, *Me* (Q_1–Q_3)

Table

The number of cells in the primary tumor microenvironment of breast cancer patients depending on the PD-L1 status according to the xCell analysis, units, *Me* (Q_1–Q_3)

Cell type		PD-L1 status		*p* (intergroup)
		negative	positive	
Naive B lymphocytes	a	0.0000 (0.0000–0.0001) *p* (a–b) = 0.0075 *p* (a–e) = 0.0323	0.0000 (0.0000–0.0001) *p* (a–b) < 0.0001 *p* (a–c) < 0.0001	0.7876
B lymphocytes	b	0.0092 (0.0000–0.0362)	0.0530 (0.0299–0.0713) *p* (b–d) < 0.0001 *p* (b–e) < 0.0001	0.0001
Ig class-switched lymphocytes	c	0.0168 (0.0000–0.0482)	0.0388 (0.0107–0.0506) *p* (c–d) = 0.0005 *p* (c–e) < 0.0001	0.0490
Plasma cells	d	0.0001 (0.0000–0.0087)	0.0008 (0.0000–0.0159)	0.9170
Memory B lymphocytes	e	0.0213 (0.0046–0.0310)	0.0000 (0.0000–0.0029)	0.0847

A comparison of B lymphocyte levels between patient groups depending on their PD-L1 status revealed that patients with PD-L1 positive status had higher levels of B lymphocytes and Ig class-switched B lymphocytes in the primary tumor microenvironment compared to PD-L1 negative patients (Fig. 2).

Assessment of B lymphocyte maturation and PD1 expression. As in the transcriptome analysis, we detected B lymphocytes in the primary tumor microenvironment in all patients. The variability of the determined population composition is shown in Fig. 3.

The assessment of CD27 (lymphocyte maturation marker) and PD1 (functional receptor molecule for PD-L1) expression showed that CD27+PD1- B lymphocytes were not found in the primary tumor tissue in all patients, regardless of the PD-L1 status in the primary tumor. CD27-PD1- B lymphocytes were the most rare (in 12.5% (2 / 16) of patients in both groups). CD27+PD1+ and CD27-PD1+ B lymphocytes were found with comparable frequency in the primary tumor tissue (81.2% (13 / 16) and 81.2% (13 / 16), respectively, $p > 0.9999$).

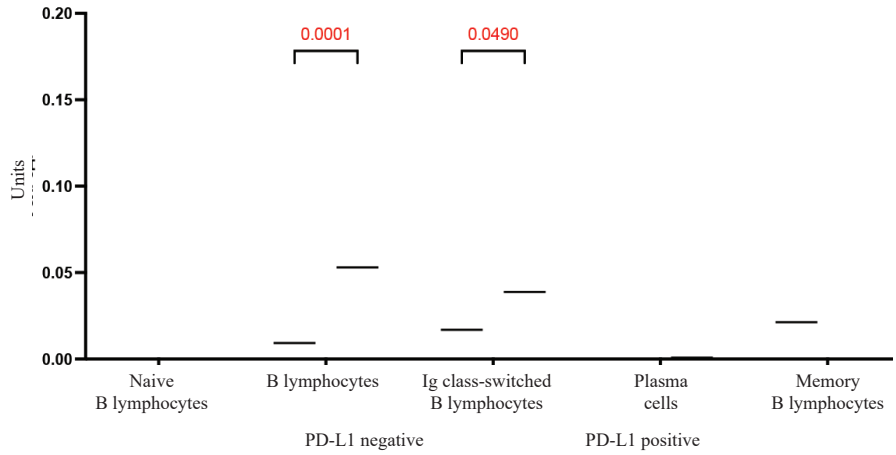

Fig. 2. B lymphocyte subpopulations in PD-L1 negative and PD-L1 positive breast cancer patients

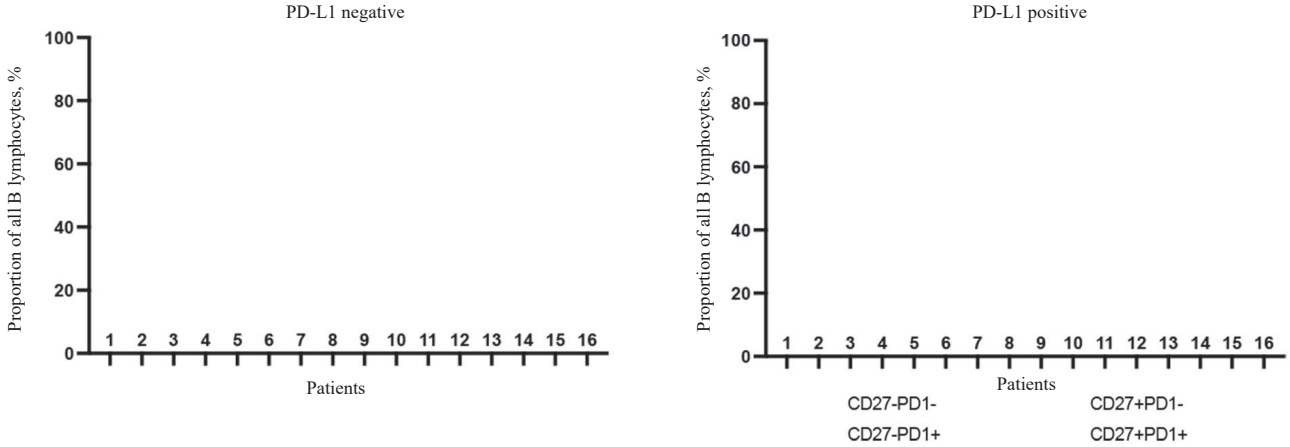

Fig. 3. Intrapersonal heterogeneity of B lymphocyte composition in PD-L1 negative and PD-L1 positive breast cancer patients

The assessment of the number of different B lymphocyte subpopulations in patients depending on the PD-L1 status showed that PD-1 positive B lymphocytes prevailed in the tumor microenvironment in PD-L1 positive patients, regardless of their maturity (Fig. 4).

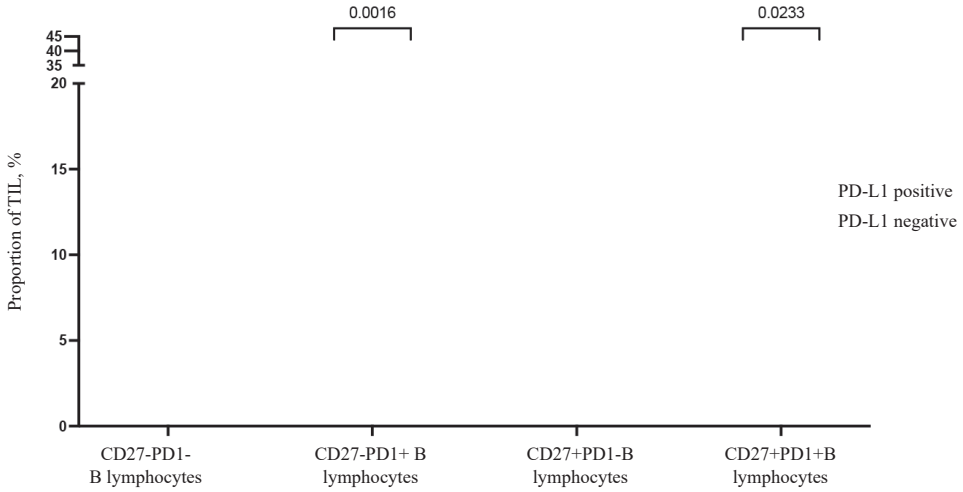

Fig. 4. The number of B lymphocytes with different combination of CD27 and PD1 expression in patients, depending on the PD-L1 status

The number of CD27+PD1+ B lymphocytes in PD-L1 negative patients was 0.09 (0.00–0.89) versus 1.67 (0.61–3.22) % in PD-L1 positive patients (p = 0.0233). The number of CD27-PD1+ B lymphocytes was 0.05 (0.00–1.53) % in PD-L1 negative patients and 1.41 (0.25–7.23) % in PD-L1 positive patients (p = 0.0016).

DISCUSSION

Inflammatory infiltration in the tumor microenvironment reflects a combination of innate immune responses and specific adaptive immune responses to various antigens: tumor, tissue and pathogenic antigens contaminating tumor tissue. The ratio of these components of immune and inflammatory responses in the microenvironment is individual in each case. B lymphocytes are a key link in the antibody genesis, a source of cytokines belonging to "proinflammatory" ("protumor") Th2 cytokines. Accordingly, the effect of the B cell link of the immune response on tumor elements is possible due to the synthesis of antibodies specific to tumor antigens. The effect depends on the type of immunoglobulins. Theoretically, both antibody-dependent cytotoxic reactions involving granulocytes and macrophages and "masking" of tumor antigens by antibodies preventing cytotoxic effects of CD8+ T lymphocytes are possible. Tumor cells are also affected by cytokines. Moreover, regardless of B lymphocyte specificity to the tumor or non-tumor antigen, activation of such cells by the corresponding antigen causes secretion of Th2 cytokines, which contributes to carcinoma progression: epithelial – mesenchymal transition, invasion, angiogenesis, and, finally, metastasis.

The results of our study allowed us to evaluate the presence and activity of B lymphocytes at different stages of functional differentiation from naive B lymphocytes and Ig class-switched B lymphocytes to memory B lymphocytes. The main results of the study are the following: 1) B lymphocytes express PD1 and PD-L1; 2) the number of differentiated forms of B lymphocytes depends on the PD-L1 status. The number of Ig class-switched B lymphocytes and memory B lymphocytes is greater when PD-L1 is expressed in the tumor infiltrate. Moreover, there are no cells without PD1 expression among CD27+ B lymphocytes. These results imply that B lymphocytes actively involved in antibody genesis (with the ability to switch from early IgM to IgG, IgA, or IgE immunoglobulins) as well as memory B lymphocytes (with the potential to develop secondary immune responses to repeatedly exposed antigens) are most likely blocked through the PD1 / PD-L1 pathway. This phenomenon corresponds to normal inflammation when there is an increase in PD-L1 expression on various cells in the foci of inflammation [5]. This prevents nonspecific damage to the tissues where inflammatory responses occur and ensures selective local activation of B lymphocytes during ligand blockade.

CONCLUSION

The results show the predominance of mature committed B lymphocytes and memory B lymphocytes capable of synthesizing immunoglobulins of different classes and cytokines belonging to type 2 immunity in the microenvironment of PD-L1 positive tumors. This may have an adverse effect, since the use of immunotherapy with anti-PD-L1 inhibitors is highly likely to activate cells with protumor potentials in the microenvironment, which ultimately will contribute to the progression of carcinomas.

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Authors contribution

Tashireva L.A. – conception and design, analysis and interpretation of the results, drafting of the manuscript. Kalinchuk A.Yu., Gerashchenko T.S., Savelyeva O.E. – conducting the research. Perelmuter V.M. – final approval of the manuscript for publication.

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10-year stability of magnetite nanopowder prepared by the exploding wire method: is it a useful feature for environment safety and biomedical applications?

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ABSTRACT

Aim. To analyze the structural, magnetic, and cytotoxic features of magnetite nanoparticles (MNPs) prepared by the exploding wire method and stored in a dark place at ambient temperature (65 ± 15% humidity, air pressure 760 ± 20 mm Hg., temperature 22 ± 4 °C) for 10 years.

Materials and methods. The properties of MNPs were analyzed by X-ray diffraction (XRD), transmission electron microscopy (TEM) and selected area electron diffraction (SAED), and vibrating-sample magnetometer (VSM). Viability of human blood mononuclear leukocytes was determined using 0.4% trypan blue staining after 24-hour culture with the nanopowder.

Results. The calculated size of the particles remained almost unchanged after 10 years of storage. The XRD and SAED patterns showed that crystallinity was preserved for 10 years. The diameter of the crystalline component of MNPs (D_{XRD}) was close to the particle size determined by TEM. It confirms high crystallinity of the tested nanoparticles. Saturation magnetization (M_S) of the MNP powder after 10 years of storage was unexpectedly higher than that of the as-prepared MNP powder. Reduced remanent magnetization (M_R / M_S) was equal for both samples within the margin of error. No cytotoxic effect of MNPs *in vitro* was detected in the long-term study.

Conclusion. No dramatic changes in the structural, magnetic, and cytotoxic features of MNPs were noted after 10 years of storage. It indicated 10-year stability of MNP powder that may be a useful feature for environment safety and biomedical applications.

Keywords: magnetite nanoparticles, ambient conditions, nanopowder stability, human blood mononuclear leukocytes, *in vitro* cytotoxicity

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Десятилетняя стабильность нанопорошка магнетита, приготовленного методом электровзрыва проводников: полезное свойство для экологической безопасности и биомедицинского использования?

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РЕЗЮМЕ

Цель. Провести анализ структурных, магнитных и цитотоксических свойств наночастиц магнетита (МНЧ), приготовленных методом электровзрыва проводников и хранящихся в защищенном от света месте при комнатной температуре (влажность 65 ± 15%, атмосферное давление 760 ± 20 мм рт. ст., температура 22 ± 4 °С) в течение 10 лет.

Материалы и методы. Свойства МНЧ изучали с помощью рентгеноструктурного анализа (РСА), просвечивающей электронной микроскопии (ПЭМ) с исследованием электронной дифракции на выбранной области (SAED) и магнитометрии с вибрирующим образцом (VSM). Жизнеспособность мононуклеарных лейкоцитов крови человека определяли окрашиванием 0,4%-м раствором трипанового синего после 24-часового сокультивирования с нанопорошком.

Результаты. Расчетный размер частиц практически не изменился после 10 лет хранения. Картины электронной и ренгеновской дифракции показали, что кристаллическая природа сохранялась в течение 10 лет. Диаметр кристаллической части МНЧ, определяемый, как область когерентного рассеивания ренгеновского излучения ($D_{ОКР.}$) был близок к размеру частиц, определенному с помощью ПЭМ ($D_{ТЕМ}$), что свидетельствует об их высокой кристалличности. Намагниченность насыщения (M_S) для порошка МНЧ после 10 лет хранения оказалась неожиданно выше, чем для свежеприготовленного порошка МНЧ. Приведенная остаточная намагниченность (M_R/M_S) была одинаковой в пределах погрешности измерений для обоих образцов. При длительном исследовании *in vitro* цитотоксическое влияние МНЧ не установлено.

Заключение. Кардинальные изменения структурных, магнитных свойств МНЧ после 10-летнего хранения не обнаружены. Сделан вывод о 10-летней стабильности электровзрывного нанопорошка магнетита, которая может быть полезна в плане его экологической безопасности и биомедицинских приложений.

Ключевые слова: наночастицы магнетита, комнатные условия, стабильность нанопорошка, лейкоциты крови человека, цитотоксичность *in vitro*

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INTRODUCTION

Since 1989, about 100 nanomedical applications and products have been approved by the US FDA and entered the global market. Since 2010, the development of nanomedicines and the number of marketed nanomedicines have significantly increased due to the resulting healthcare benefits [1, 2]. Magnetic nanoparticles (MNPs) are of interest due to their unique magnetic properties and are widely used in different fields, such as nanomedicine, engineering, agriculture, energy, and environmental remediation [3].

MNPs are a class of materials with unique physical and chemical properties, which may affect the environment [4] and biological systems. Therefore, determining hazards and risks associated with their application is relevant. Risk assessment is crucial for developing recommendations on MNP effects and application [5]. Circulation of nanochemical agents in the industry, environment, and among customers, as well as their wastes and storage time are essential for understanding the balance between efficiency and safety of nanotechnologies.

In this regard, more attention should be paid to concerns related to long-term stability of magnetic iron oxide nanoparticles *ex vivo*, since nanoscale magnetite (Fe_3O_4) oxidizes to maghemite (γ-Fe_2O_3) in ambient conditions. However, the duration of this process has not been well described. There are very few studies focused on MNP aging, for example, one study showed that the process lasts 18 months in an aqueous suspension [6]. We analyzed the structural, magnetic, and cytotoxic properties of MNPs prepared by the exploding wire method and then stored in a dark place at ambient temperature (65 ± 15% humidity, air pressure 760 ± 20 mm Hg., temperature 22 ± 4 °C) for 10 years.

MATERIALS AND METHODS

The nanopowder was obtained by V.S. Sedoi (the Institute of High Current Electronics, Siberian Branch of the Russian Academy of Sciences, Tomsk, Russia) by the exploding wire method (EWM) in 2007. A detailed methodology and experimental setup used in the present study for structural and magnetic evaluation were described earlier [7, 8]. EWM makes it possible to obtain nanoparticles of various compositions, including metallic iron (α-Fe) and its oxides with good crystallinity, which is confirmed by the high saturation magnetization value of the obtained nanoparticles [9].

The crystal structure and phase composition of the samples were examined by X-ray diffraction (XRD). The morphology and microstructure were determined by transmission electron microscopy (TEM) with selected area electron diffraction (SAED). Magnetic properties were studied using a vibrating sample magnetometer (VSM) at room temperature (295 K) in a field range of 1.1 T.

The cytotoxicity of MNPs was tested *in vitro* using human blood mononuclear leukocytes (MNLs) collected from the venous blood of healthy volunteers (Protocol No. 948 of 09.02.2009; the local Ethics Committee of Siberian State Medical University, Tomsk, Russia; Protocol No. 5 of 16.05.2016; the local Ethics Committee of Immanuel Kant Baltic Federal University, Kaliningrad, Russia) [10].

Aseptic MNP ferrofluid in isotonic (0.9%) saline (30 mg / l, pH = 6.9) was prepared by ultrasonication for 30 min at a power output of 100 W. No stabilizers (sodium citrate, chitosan, etc.) were added because of their possible effect on the cells. Moreover, even stabilized ferrofluids demonstrated unequal distribution of MNPs in the cell culture [11].

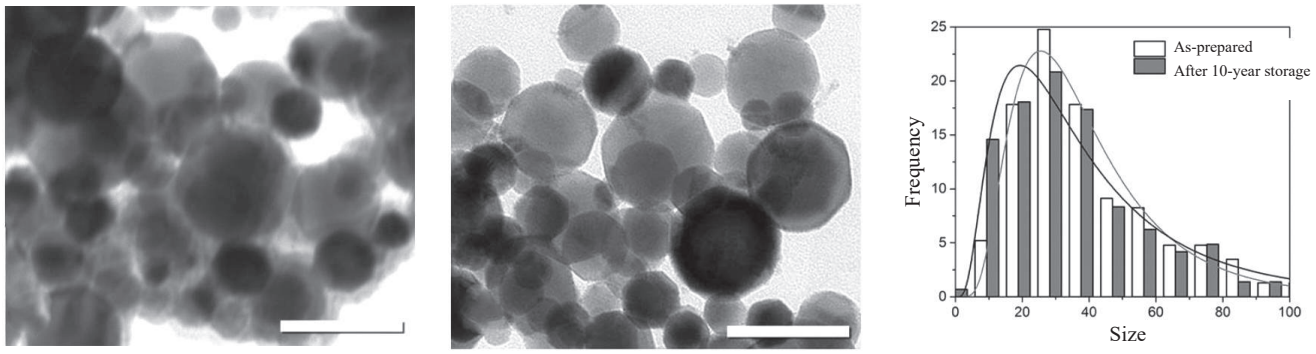
The MNL suspension in plastic tubes contained 1×10^6 viable cells per 1 ml of the synthetic nutrient medium (Sigma-Aldrich, USA) containing RPMI-1640, 10% fetal bovine serum, 50 mg / l gentamicin, and 280 mg / l L-glutamine. This suspension was mixed with the MNP ferrofluid immediately after ultrasonication at a ratio of 10 : 1 to preserve the pH value of 7.2–7.3 in the cell culture. The final MNP concentration in the cell culture was ten maximum tolerated doses (10 MTDs = 3 mg / l) of MNPs in relation to iron ions. One MTD of iron in water is equal to 0.3 mg / l. Cell viability was calculated on the Countess Automated Cell Counter (Invitrogen, USA) using 0.4% trypan blue solution (Invitrogen, USA) according to ISO 10993-5 after 24 hours of culturing at 5% CO_2, 100% humidity, and 37 °C. The main antigen profile of MNLs (CD45, CD3, CD14) was analyzed by flow cytometry [10].

The results were analyzed using Statistica 13.3 for Windows. The data were presented as the mean and the standard deviation $M \pm SD$. Due to non-normal distributrion of variables (the Shapiro – Wilk test), the Mann – Whitney U-test (p_U) was used to evaluate statistical differences in the samples.

RESULTS AND DISCUSSION

The values for average particle size distribution (D) are presented in Table 1. According to TEM, MNPs have a spheroid or polyhedral shape with rounded edges (Fig.

1). TEM images for the as-prepared samples performed in 2007 showed that the particles had smoothed edges, which may be attributed to poorer crystallinity of the surface or to the fact that the equipment used 10 years ago had lower resolution. TEM images were processed using ImageJ software. To analyze MNP size distribution, the obtained distribution was fitted with the lognormal distribution [11].



a *b* *c*

Fig. 1. TEM images of iron oxide powder: *a* – as-prepared; *b* – after 10 years of storage in ambient conditions, *c* – histogram of size distribution: scale bar is 100 nm.

The calculated MNP size remained almost the same after 10 years of storage. MNPs demonstrated lognormal size distribution with similar average diameters for the as-prepared and long-stored samples (Table 1).

The SAED pattern showed that crystallinity persisted for 10 years (Fig. 2, *a*). Bright rings are formed by spots related to the (111), (220), (311), (400), (422), (511), and (440) axis of the face-centered cubic structure of Fe_3O_4. One weak intensity ring marked as 110_a was associated with a small content of metallic a-Fe.

The quantitative phase analysis was performed with Rietveld refinement (Table 1). The results of the XRD measurements of iron oxide powder demonstrated that the predominant phase in the particles was magnetite (X-ray scan 04-015-3102), with a value of approximately 96−97 wt.%. In addition, a small amount of metallic a-Fe (X-ray scan 04-014-0360) was observed with a value of approximately 2−3 wt.% (Fig. 2, *b*, Table 1).

Table 1

Phase composition, particle size, and magnetic properties of the MNP powder, *M (SD)*

Year	Phase composition, wt.%			Structural properties		Magnetic properties (295 K)	
	Fe_3O_4/ γ-Fe_2O_3	α-Fe	Unidentified phases	D_{XRD}, nm	D_{TEM}, nm	M_R/M_S	M_S, Am²/kg
2007	96	3	~1	31(3)	34 (0.6)	0.14 (0.01)	71.1 (0.9)
2017	97	2	~1	32(4)	33 (0.7)	0.16 (0.01)	79.8 (0.4)*

* $P_U < 0.05$; the measurements were carried out in Tomsk in 2007, as well as in Tomsk and in Kaliningrad in 2017.

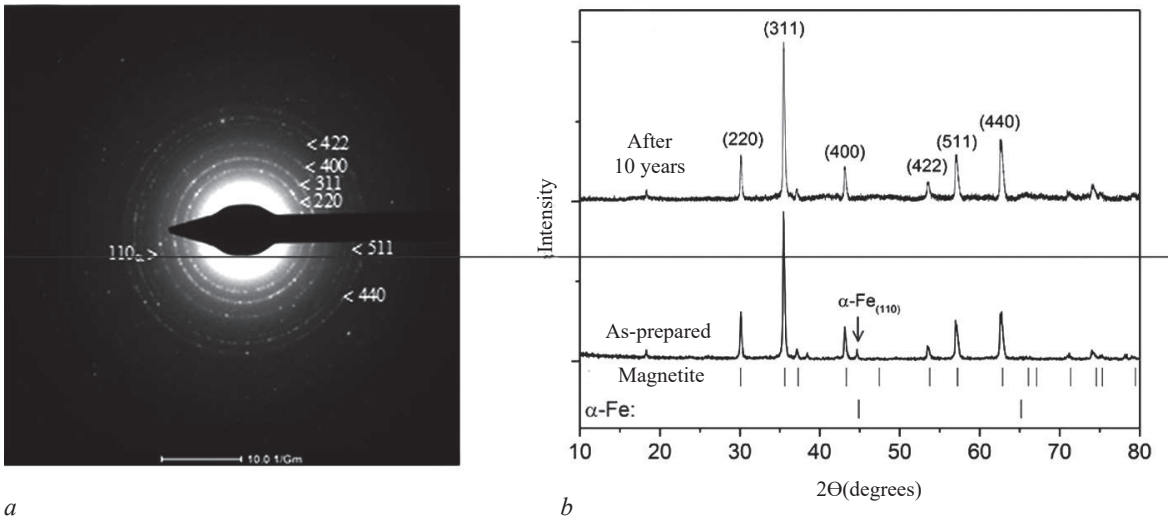
It should be noted that two ferrimagnetic iron oxides magnetite and maghemite both have an inverse spinel structure with similar lattice constants, and due to peak broadening at the nanoscale, they cannot be distinguished with conventional X-ray diffraction techniques [12]. The average size of the coherent scattering domain (D) was calculated with the Scherrer equation (1).

$$D_{XRD} = k\lambda / (\beta \times \cos\theta),$$

where the shape factor *k* is 0.9 for spherical shape, λ is the wavelength of Cu Kα radiation (1.54178 Å) and β is the full width at half-height [13]. For nanoparticles with good crystallinity, this size was close to the particle size determined by TEM (Table 1).

The saturation magnetization (M_S) for the aged MNPs calculated according to Fig. 3 was slightly greater than that for the as-prepared MNPs (Table 1). Reduced remanent magnetization (M_R / M_S) was equal for both samples within the margin of error.

Two magnetic iron oxides, magnetite and maghemite, have different values of saturation magnetization ($M_S^{Fe_3O_4} = 92-100$ and $M_S^{\gamma\text{-}Fe_2O_3} = 60-80$ Am / kg [14]).



a *b*

Fig. 2. SAED pattern of MNP powder stored for 10 years (*a*); XRD pattern of the as-prepared sample and the sample after 10 years of storage (*b*) in ambient conditions

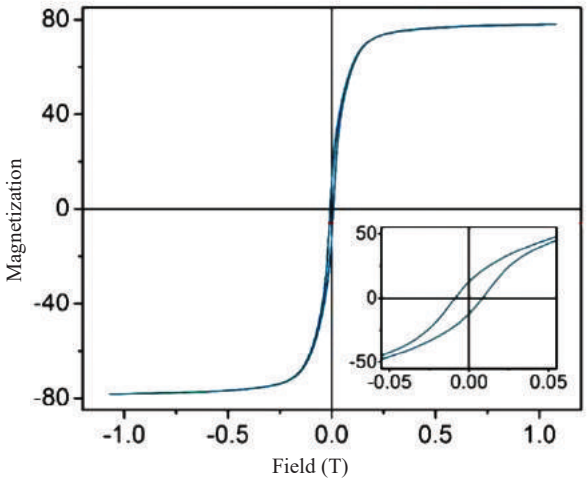
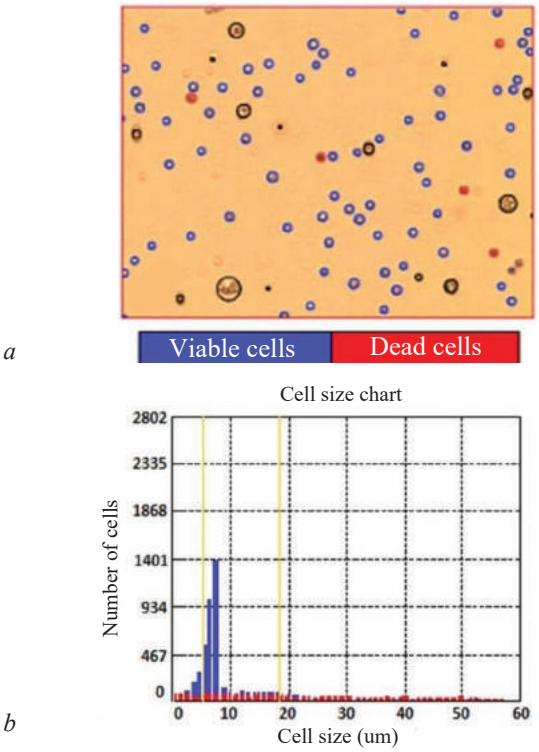


Fig. 3. Hysteresis loop of MNPs powder at T = 295 K after 10 years of storage in ambient conditions

Obtained M_S values were closer to the maghemite phase, however, some decrease in magnetization can be also related to the size effects (e.g. the presence of a fraction of canted surface spins or antiphase boundaries) [15, 16].

The cytotoxic effect of MNPs was not detected *in vitro* using a cell counter (Fig. 4) for any year of a long-term study (Table 2). The mean number of living MNLs within the min – max spectrum of diameters (7–18 μm) did not differ significantly after adding the same MNPs at 10 MTDs in 2007 and 2017.

A total of 93–95% of MNLs with sizes of 7–11 μm (Fig.4) expressed CD45CD3 T cell antigens. Large cells presented CD45CD14 markers of monocytes / macrophages in 5–7% of the cases, which did not depend on MNP use and year.



a *b*

Fig. 4. Calculation of cell viability (a) and size (b) using the Countess Automated Cell Counter

Table 2

In vitro* viability of MNLs after 24-hour co-culture with MNPs at 10 MTDs, *M* ± *SD

Nanopowder	Number of viable (not stained with tryptan blue) cells, %, n = 5	
	Control cell cultures (without MNPs)	Cell cultures with MNPs
As-prepared	86.2 ± 1.62	85.8 ± 3.02
After 10 years of storage	87.5 ± 2.06	85.6 ± 2.71

CONCLUSION

The 10-year stability of the structural, magnetic, and cytotoxic properties of electroexplosive magnetite nanopowder, which was stored in the dark place at ambient temperature, can be considered a useful property to ensure a balance between its environmental safety and biomedical properties as an inorganic carrier for the diagnosis and treatment of cancer and a wide variety of other diseases.

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Authors contribution

Khlusov I.A., Rodionova V.V., Litvinova L.S. – conception and design, interpretation of the results. Omelyanchik A.S. – carrying out of the physical part of experiments, drafting of the manuscript. Shupletsova V.V., Khaziakhmatova O.G., Yurova K.A. – carrying out of the biological experiments. Norkin I.K. – statistical analysis of the data.

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Microbiota: its contribution to carcinogenesis and immunity in the lungs

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ABSTRACT

Microbiota (an assembly of bacteria, protists / archaea, fungi, and viruses inhabiting a human body) is currently of great interest for science. It is determined by an association between changes in microbiota composition and malignant transformation in different organs. Lungs have long been considered sterile or free from bacteria; however, due to development of next-generation sequencing, this statement has been reconsidered. The metagenomic approach allowed to identify microorganisms at molecular level both in healthy lung tissues and in malignant ones.

The next stage of research is investigation of the effects of microbiota on homeostasis and immune stability in the lungs. The analysis of lung microbiota based on 16S rRNA gene sequencing revealed that microbiota of healthy lungs is mainly presented by bacteria of the phyla *Bacteroidetes, Firmicutes, Proteobacteria,* and *Fusobacteria*. In lung cancer, an increase in the number of bacteria of some certain genera and a decrease in microbiota diversity on the whole are noted. Dysbiosis facilitates reproduction of pathogens and development of lung diseases. It was detected that under normal conditions, microbiota maintains resistance of the lungs to bacterial colonization and plays a crucial role in providing a balanced immune response in this organ.

Keywords: metagenomics, microbiota, lungs, lung cancer,16S rRNA, immunity

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Микробиота: вклад в канцерогенез и функционирование иммунной системы легких

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РЕЗЮМЕ

Микробиота (совокупность бактерий, простейших/архей, грибов, вирусов, обитающих в организме человека) и микробиом (их совокупный геном) являются предметом активных научных исследований. Особый интерес вызывает взаимосвязь изменений состава микробиоты и злокачественной трансформации различных органов. Легкие долгое время считались стерильным органом, однако это представление было пересмотрено благодаря развитию технологий секвенирования нового поколения. Метагеномный подход позволил идентифицировать микроорганизмы на молекулярном уровне в здоровых тканях легкого и в опухолях.

Следующим шагом стало выявление разнообразных аспектов влияния микробиоты на гомеостаз легочной системы и поддержание иммунитета. Анализ результатов исследований микробиоты легочной системы, основанных на секвенировании генов 16SpРНК, позволил установить, что микробиота здоровых легких представлена в основном бактериями, принадлежащими к типам *Bacteroidetes, Firmicutes, Proteobacteria* и *Fusobacteria*. При развитии рака легкого отмечено значительное повышение численности бактерий определенных родов и в целом снижение разнообразия микробиоты. Дисбиоз способствует активному размножению патогенов и развитию негативных состояний легочной системы. Установлено, что в норме легочная микробиота обеспечивает устойчивость к заселению легких болезнетворными микроорганизмами и играет важную роль в обеспечении сбалансированного иммунного ответа в данных органах.

Ключевые слова: метагеномика, микробиота, легкие, рак легкого,16SpРНК, иммунитет

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INTRODUCTION

Lung cancer (LC) is currently recognized as one of the most widespread causes of cancer mortality in both men and women. Less than 20% of LC patients live longer than 5 years after the diagnosis was established [1]. At present, it is understood that lungs are not sterile and free from bacteria. The results of recent surveys show that lung microbiota can affect homeostasis of human respiratory system and play a role in LC development or in formation of metastases in the lungs as a consequence of primary cancer in other organs. Lung microbiota dysbiosis affects the risk of developing cancer at several levels, for example, by causing chronic inflammation or activation of oncogenes. Studying the effects of human respiratory microbiota on LC development and therapy effectiveness may be crucial in assessing the risk of pathology and developing a strategy for its treatment.

An assembly of microorganisms (bacteria, archea, fungi, viruses, protists) living in the human body is called microbiota, and their combined genome is called microbiome [2, 3]. Currently, the relationship between microbiota and cancer is being actively investigated. Most experimental studies are devoted to revealing the pathogenic properties of bacteria. For example, bacterial toxins can disrupt cell cycle by interfering with the synthesis of proteins responsible for DNA repair, cell division, and apoptosis. Bacteria affect the effectiveness of immunotherapy and the development of host immune responses against cancer cells [4].

Until recently, it was impossible to study bacteria in the lungs using conventional culture methods. Modern next-generation sequencing (NGS) makes it possible to effectively detect bacterial DNA [5–9]. This approach allowed researchers to identify

microorganisms at the molecular level in complex biological samples. Depending on the bacterial kingdom of interest, primers specific to conserved regions of genomes are used: 16S rRNA and 18S rRNA for bacteria and archaea, ITS1–ITS2 for fungi, V4–V9 regions of 18S rRNA for protists. Shotgun sequencing is used to identify viruses after primary extraction of viral particles [10]. A combination of conventional and novel methods of analysis, 16S rRNA gene sequencing and matrix-assisted laser desorption / ionization, and advancements in bioinformatics analysis of big repositories over the last five years made it possible to perform whole-genome sequencing of microorganisms and identify new species [11]. Interestingly, conventional culture methods are more effective in detecting the species of *Mycobacterium* genus [12].

Researchers are still discussing the effects of microbiota on lung homeostasis and its role in maintaining immunity**.** The aim of this review was to summarize the results of studies on evaluating microbiota contribution to the functioning of immunity and the development of LC published over the past 10 years.

MICROBIOTA OF HEALTHY LUNGS

Bacteria colonizing the human body belong mainly to *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria,* and *Cyanobacteria* phyla [13–16]. Currently, it was determined that the numbers of bacterial and human cells in the human body are equal. The number of bacteria inhabiting healthy human lungs is estimated from hundreds of thousands to hundreds of millions per 1 ml of lung volume [17].

A sufficient amount of information was obtained about the characteristics of lung microbiota depending on certain physiological conditions of the host organism. It was noticed that functional stability is provided by bacterial taxa that make up "healthy microbiota". Thus, under normal conditions *Proteobacteria, Firmicutes, Fusobacteria,* and *Bacteroidetes* phyla are the largest in number [18, 19]. They also include *Pseudomonas, Streptococcus, Prevotella, Veillonella, Haemophillus, Neisseria* genera, inhabiting the respiratory tract. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria,* and *Fusobacteria*, as a rule, dominate in healthy lungs [20, 21]. However, it should be noted that when certain conditions change, some of the listed taxa may perform destructive functions. In general, in the absence of pathological conditions in the lungs, spatial differences in microbiota composition are not observed.

In most healthy people, oral commensals belonging to *Prevotella*, *Veillonella,* and *Streptococcus* genera are also found in lung microbiota, probably due to swallowing of the pharynx content, although these bacteria are not observed in all healthy individuals [22, 23]. Lung microbiota is also distinguished into pneumotypes according to quantitative and qualitative characteristics of specific taxa. The first group includes microbiome with a high bacterial count enriched with bacteria from the oral cavity, such as *Prevotella* and *Veillonella* (supraglotic predominant taxa (SPT)). The second group includes microbiome with a low content of *Prevotella* and *Veillonella* and trace amounts of bacteria from the environment, such as *Acidocella* and *Pseudomonas* (background predominant taxa (BPT)). It was shown that the SPT-pneumotype corresponds to a local Th17 immune response. Its functioning determines the immune status in normal and pathological conditions [24]. The relationship between pneumotypes and a risk of developing lung pathology is the focus of many studies. Besides, there are studies that confirmed that microbiota of healthy lungs differs from that of the oral cavity and other parts of the respiratory system and consists mainly of *Proteobacteria* (up to 60%).

LUNG MICROBIOTA IN LUNG CANCER

Malignant transformation in LC promotes structural changes in the microbiota composition. In LC patients, *Actinomyces* and *Peptostreptococcus* genera are most often detected in the lower respiratory tract. Pathogenesis is also associated with the activity of oral cavity bacteria (*Streptococcus* and *Wechsler*), which are involved in triggering the ERK and PI3K signaling pathways. Infections caused by *Mycobacterium tuberculosis* and *Helicobacter pylori*, associated with inflammation, enhance oncogenesis [25–27]. *Eubacterium xylanophilum, Eubacterium eligens,* and *Clostridium* also contribute to the most acute course of LC, their increased number is associated with the development of small cell lung cancer. Certain taxa (*Acidovorax*), in addition to cancers, can be involved in the development of other lung diseases. In the respiratory tract, *Propionibacterium* members contribute to the development of mild LC; however, their antitumor potential was demonstrated on laboratory mice [28]. Currently, sufficient data have been collected on the hypothetical effect of respiratory microbiota on LC development [29].

In LC, *Firmicutes* and *TM7* phyla and also *Eillonella, Megasphaera, Atopobium*, and *Selenomonas* genera are most often detected in lung microbiota. *Atopobium* and *Selenomonas* cause the development of milder oncogenic processes, and *Megasphaera* contributes to the development of acute LC. Representatives of this taxon, along with *Veillonella*, can be used as specific LC biomarkers for diagnosis and therapy of this pathology. *Filifactor* and *Treponema* genera were determined as significant markers of LC development using bronchoalveolar lavage fluid as a study material [30]. The number of *TM7* phylum members is increased in COPD and also in LC, which indicates possible development of oncogenic processes in case of increased inflammation.

As a result of metagenomics studies on LC using different material in combination with specific conditions, a significant increase in specific bacterial taxa and a simultaneous decrease in microbiota diversity were detected (Table).

Table

Bacterial communities detected in patients with lung cancer

Bacteria	Sample size	Sample type	References
H .influenzae *Enterobacter spp* *Escherichia coli*	216	Airway endoscopy	[31]
Granulicatella *Abiothrophia* *Streptococcus*	16	Sputum samples	[35]
Granulicatella *Streptococcus* *Mycobacterium*	10	Sputum samples	[36]
Acidovorax	176	Lung tissue	[38]
Brevundimonas *Acinetobacter* *Propionibacteium*	103	Bronchoalveolar lavage	[39]
Lactobacillus rossiae, Bacteroides pyogenes, Paenibacillus odorifer, Pseudomonas entomophila, Magnetospirullum gruphiwaldense	47	Bronchoalveolar lavage	[41]

The study of airway endoscopy material made it possible to evaluate pathogenic properties of gram-negative *H.influenzae*, *Enterobacter spp.*, *Escherichia coli* and gram-positive *Mycobacteria* [31]. In another study of bronchoalveolar lavage fluid, S.H. Le et al. found an increase in *Veillonella* and *Megaspahaera* genera during the development of LC [32]. Currently, researchers have no clear understanding of the role of *Streptococcus* and *Staphylococcus* in LC carcinogenesis. This may be due to difficulties of identifying other bacteria or due to the fact that bacteria may play a different role depending on a variety of conditions. The discrepancies in the data may be affected by lifestyle factors, specificity of environmental pollution (for example, the use of coal for heating), smoking, features of sampling the material for analysis and other factors [33]. It should be noted that an increase in *Streptococcus* members is typical of lung cancer [34].

It is known that exposure to chemical pollutants, in particular polycyclic aromatic hydrocarbons (PAHs), increases the risk of developing LC. H.D. Hosgood et al. examined the microbiota composition in non-smoking women who used coal as fuel at home. An increased content of *Granulicatella, Abiothrophia*, and *Streptococcus* was detected in the sputum samples [35]. An increase in *Granulicatella* (*Granulicatella adiacens*) was associated with LC progression [36]

Smoking can multiply the risk of LC formation. Currently, a lot of data have been collected about the molecular mechanisms underlying tobacco smoking. Smoking may contribute to the development of dysbiosis in different parts of the body, causing many diseases (asthma, COPD, and LC) [37]. Tobacco smoke directly interacts with the respiratory epithelium and contributes to impairment of immunological barriers. As a result, taxonomic composition and phylogenetic diversity of lung microbiota change (Fig. 1). The variability of *Firmicutes / Bacteroidetes* proportion in non-smoking / smoking patients should be taken into account to understand the role of microbiota in this case.

In context of studying microbiota, a hypothesis was put forward about the synergistic effect of somatic mutations and disruption of the epithelial barrier due to tobacco smoking in the development of LC. A study was conducted to understand the effect of *TP53* gene mutations on the composition of lung microbiota in in order to prove this hypothesis [38]. Initially, when

comparing the samples obtained from tumor tissues with those obtained from healthy ones, an increase in the number of *Proteobacteria* and a decrease in the number of *Firmicutes* were detected. The presence of *Acidovorax, Ruminococcus, Oscillospira, Duganella, Ensifer,* and *Rhizobium* genera was associated with smoking. In particular, members of *Acidovorax* were the most common taxa in smokers. The association of *Acidovorax* members with LC development was revealed in the presence of mutations in the *TP53* gene.

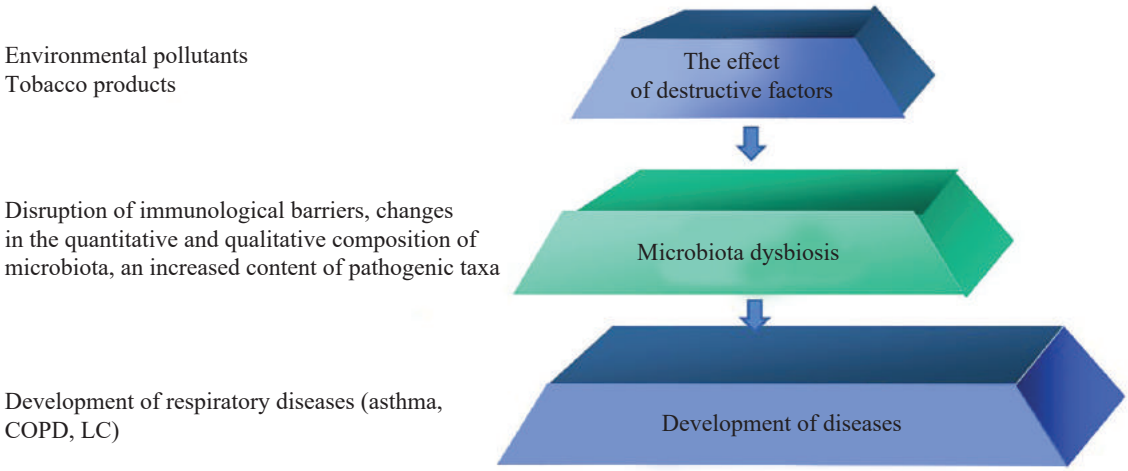

Fig. 1. Development of microbiota dysbiosis

An important issue is differences in microbiota depending on the histopathological type of LC. S. Gomes et al. revealed an increase in the proportion of *Brevundimonas, Acinetobacter,* and *Propionibacteium* taxa in patients with lung adenocarcinoma [39]. The presence of *Enterobacter* was characteristic of squamous cell LC. The development of non-small cell LC may also be accompanied by an increase in the activity of intestinal microbiota. In one study, bacteria of *Phascolarctobacterium* genus were strongly associated with the development of squamous cell LC [40]. *Phascolarctobacterium faecium* and *Phascolarctobacterium succinatutens* species detected in this study belong to microbiota in the gastrointestinal tract. Despite their unique properties, lung microbiota in tumors may have similarities with healthy tissues. At the same time, the presence of such rare bacterial species as *Lactobacillus rossiae, Bacteroides pyogenes, Paenibacillus odorifer, Pseudomonas entomophila,* and *Magnetospirullum gruphiwaldense* was observed in non-small cell LC [41].

Patients with LC are most often characterized by a decrease in the alpha diversity of lung microbiota [42]. A similar phenomenon was observed in the analysis of lung adenocarcinoma [43]. Regarding beta diversity, there are data about the absence of differences between malignant and healthy tissues. When assessing the overall diversity of microbiota, an increase in the Shannon diversity index was also noted in LC patients compared with healthy individuals.

MICROBIOTA AND IMMUNITY IN THE LUNGS

Studies of the past decade showed that lung microbiota maintains resistance of the lungs to bacterial colonization and plays an important role in providing a balanced immune response in the lungs. The composition of lung microbiota and its relationship with human immunity change with age, probably due to environmental effects. The evolution of these relationships leads to the development of regulatory processes that determine resistance to host antigens and non-dangerous agents and ensure exclusion of pathogens and transformed cells [44].

The pathogenesis of many diseases can largely be determined by specific interactions of bacterial communities from different ecological niches of the human body (Fig.2). Interaction of microbiota components from different parts of the body occurs during circulation of metabolic products, proinflammatory cytokines, and other signaling molecules. Bacterial translocation is an important property which is manifested by migration of viable resident bacteria from one niche to the other. In this regard, hypotheses were formed about axes of interaction between microbial communities.

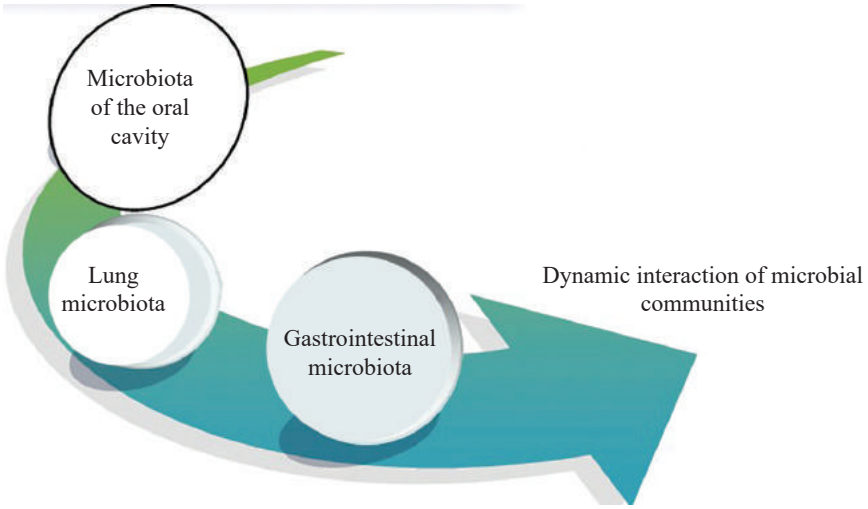

Fig. 2. Interaction between microbial communities from different ecological niches of the host organism

These include the "microbiota – brain – gut", "microbiota – gut – liver", and "microbiota – gut – skin" axes [45–47]. Despite the fact that these hypotheses are largely contradictory, nevertheless they reveal some features of the microbiota effect on physiological processes. This is mainly associated with the activation of innate and adaptive immunity mechanisms.

Lung microbiota closely interacts with other niches of the host organism. Therefore, the development of pulmonary diseases can be determined by impaired stability of gut microbiota composition. Lung microbiota and gut microbiota are currently thought to function together, refuting previous ideas about the presence of a "barrier" between them. Gut microbiota stimulates production of various regulatory cytokines and maturation of T and B cells, which provides enhanced protection of the mucous membrane. This effect not only persists in the gut, but also spreads to other mucous membranes through lymphatic and hematopoetic systems, affecting the immune response in remote organs [48].

Gut microbiota is involved in synthesis of biologically active molecules (mostly short-chain fatty acids and vitamins), which can reduce inflammatory processes. In particular, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* express anti-inflammatory interleukin 10 (IL-10) and an IL-12 inhibitor, which can stop the severe course of allergic asthma. Since malignant transformation is associated with inflammation, inhibitory properties of *Faecalibacterium prausnitzii* may have therapeutic effects on the development of LC. This feature was demonstrated on cancer A549 cell line with a decrease in the expression of proinflammatory cytokines (IL-1, TGF-B2, IL-1RA) [49]. According to the results of these studies, it becomes possible to form the hypothesis about the "microbiota – gut – lungs" axis to reveal the etiology of pulmonary diseases.

Microaspiration and aspiration are the most likely mechanisms in the association between gut microbiota and lung bacteria. The products of bacterial metabolism in the gastrointestinal tract can affect the intensity of differentiation of specific immunity components: T cells, regulatory T cells, and Th17s [50]. As a result, the immune response and systemic inflammation enhance, which reflects the way microbiota affects adaptive immune homeostasis in the development of diseases. There are also ways to transmit signals from the gastrointestinal tract to the pulmonary system through the bloodstream, which can affect the stability of respiratory microbiota composition [51]. Further research is needed to confirm the hypothesis about these mechanisms.

Metagenomics studies showed that the manifestation of abnormal immune activity occurs due to a decrease in the number of commensal bacteria, which have properties beneficial for the body. On the contrary, reproduction and activity of pathogens increase; *Gammaproteobacteria* use by-products of inflammatory responses for their growth [52]. Studying the features of lower respiratory tract microbiota composition revealed that pathological processes are largely associated with a decrease in the number of *Bacteroidetes* in healthy microbiota and a shift towards the spread of *Gammaproteobacteria.* Experimental studies conducted on humans and laboratory animals made it possible to determine members of this taxon as pulmonary pathogens [53].

As a part of the pulmonary system, alveolar macrophages and resident dendritic cells, as well as other components of immunity are a primary barrier for pathogenic microorganisms. They act as important mediators of immune responses in the lungs and are activated only if stimulated by harmful bacteria. Macrophages and dendritic cells stimulate division of regulatory T cells involved in the implementation of acquired immune responses. In addition, their crucial property is the ability to secrete signaling molecules: prostaglandin E2, tumor growth factor beta (TGF-B), and IL-10, which contributes to maintenance of homeostasis [54]. Performing the functions of antigen-presenting cells, alveolar macrophages, dendritic cells, and pulmonary epithelium cells provide recognition of pathogenic components (mainly of microbial origin) through a system of pattern recognition receptors (PPR). Activation of these receptors triggers subsequent expression of signaling molecules.

γδ T cells are important effectors and regulators of the innate immune response to pulmonary infections [55]. It was shown that inhaling non-pathogenic bacteria that do not cause dysbiosis or infections promotes activation of these cells, which prevents development of the abnormal inflammatory response. These cells also play a protective role against allergies [56–58]. Using laboratory mice, it was shown that airway colonization by certain bacterial strains in newborns protects against acute allergic reactions in the respiratory tract [59–61]. These and other studies convincingly demonstrate that a contact of the host organism with bacteria at early stages of development is crucial for formation of full-fledged and functional immunity in the lungs [62].

The development of LC due to changes in lung microbiota may be caused either by increased sensitivity of the immune system leading to chronic inflammation or by a disrupted mechanism of pathogen recognition. Sometimes healthy microbiota may contribute to formation of an environment favorable to malignant transformation of lung tissue cells. For example, some bacteria may contribute to colonization of the lung tissue by metastatic cancer cells. It was shown that local application of antibiotics reduces formation of metastases, which is associated with modulation of the immune response. This also suggests that bacteria should be used as therapeutic tools with caution.

CONCLUSION

Despite advancements in identifying the features of human microbiota, this technique has some limitations. For example, this method makes it possible to identify microorganisms mainly only at the genus level, since the analysis of short sequences of bacterial genomes is available. As an alternative, a method of whole genome sequencing was developed, which makes it possible to identify species in the microbiota. Despite the advantages of both approaches, they can only detect dominating species in the population.

Given that the lungs are always affected by upper respiratory tract bacteria and by the environment, it can be assumed that microorganisms promoting normal state of the human body sometimes can contribute to oncogenic cell transformation or development of other pathologies. Information about changes in lung microbiota and the influence of external factors on these processes will undoubtedly contribute to a better understanding of LC etiology, identify new targets for therapy, and help elaborate new immunotherapeutic approaches to the treatment of lung pathologies.

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Diastolic heart failure: boundaries of term application

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ABSRACT

Important changes regarding the understanding of the pathogenesis of chronic heart failure (CHF) marked the beginning of the millennium, and its first decade was called the decade of diastology. Even though numerous studies convincingly proved that deterioration of the left ventricular (LV) filling pressure often precedes impairment of its systolic function and a number of factors affect (especially at the onset) mainly the diastolic function without changing the conditions of blood ejection, modern classifications and approaches to CHF treatment are primarily based on the results of LV ejection fraction (EF) assessment.

In recent years, diastolic heart failure (DHF) has been often overlooked and replaced by the ambiguous term "CHF with preserved EF". However, sometimes authors use the term DHF extensively, since CHF based on myocardial insufficiency develops only via two mechanisms (systolic and / or diastolic dysfunction), and excluding one of the mechanisms allows to identify the underlying one. The term DHF can be used in clinical practice and cannot be replaced by the diagnosis of CHF with preserved EF. CHF with preserved EF is a broader concept which includes a full spectrum of cardiovascular diseases, complicated by the development of CHF without depression of the global LV contractility and requiring differentiated approaches to therapy. In addition, the results of repeated studies on LVEF in many patients may require reclassification of this CHF phenotype, which is established following the analysis of the baseline value of global LV contractility. We join M.R. Zile in the appeal to stop discriminating against the term "DHF" and present the boundaries of its correct application.

Keywords: left ventricle, systole, diastole, heart failure with preserved ejection fraction, diastolic heart failure, diastology, visualization of the heart

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Диастолическая сердечная недостаточность: границы применения термина

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РЕЗЮМЕ

Начало тысячелетия ознаменовалось важными изменениями, касающимися представлений о патогенезе хронической сердечной недостаточности (ХСН), а его первое десятилетие стали именовать декадой диастологии. Однако несмотря на многочисленные работы, в которых было убедительно доказано, что ухудшение наполнения левого желудочка (ЛЖ) нередко предшествует нарушению его систолической функции и целый ряд факторов воздействует (особенно в дебюте) преимущественно на диастолическую функцию сердца, не изменяя условия выброса крови, современные классификации и подходы к терапии ХСН в первую очередь основаны на результатах оценки значения фракции выброса (ФВ) ЛЖ.

В последние годы о диастолической сердечной недостаточности (ДСН) нередко незаслуженно забывают, подменяя неравнозначным термином «ХСН с сохраненной ФВ». Вместе с тем иногда приходится сталкиваться с расширенным применением термина ДСН авторами, исходящими из того, что ХСН, в основе которой лежит миокардиальная недостаточность, развивается только по двум механизмам (систолическая и (или) диастолическая дисфункция) и простое исключение первого может без оговорок рассматриваться в качестве подтверждения второго. Термин ДСН имеет право на применение в клинической практике и не может быть заменен диагностическим заключением «ХСН с сохраненной ФВ», так как последняя является более широким понятием, охватывающим весь спектр заболеваний сердечно-сосудистой системы, осложняющихся развитием ХСН без депрессии глобальной контрактильной функции ЛЖ и требующих применения дифференцированных подходов к терапии. К тому же результаты повторных исследований значения ФВ ЛЖ у многих пациентов могут потребовать реклассификации этого фенотипа ХСН, установленного на основании анализа исходной величины обсуждаемого индикатора глобальной сократимости ЛЖ. Авторы лекции присоединяются к известному призыву M.R. Zile прекратить дискриминацию термина ДСН и представляют границы его корректного применения.

Ключевые слова: левый желудочек, систола, диастола, сердечная недостаточность с сохраненной фракция выброса, диастолическая сердечная недостаточность, диастология, визуализация сердца

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Chronic heart failure (CHF) and the conceptual interpretation of its pathogenesis have been studied for a long time. However, despite the fact that leading cardiologists of Old and New Worlds repeatedly attempted to give a detailed definition of CHF, not a single definition was generally accepted. In our opinion, attention should be paid to the laconic and at the same time meaningful definition of the experts from the American College of Cardiology and the American Heart Association, which essentially has not changed much since 2001: "a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood" [1].

There are two points of great importance in the presented definition. Firstly, the authors, as well as the experts from the European Society of Cardiology [2], rightly consider CHF not as an independent nosological form of disease, but as a syndrome, which prompts the doctor to identify the underlying cause of heart failure in every clinical case, which is fundamental for the correct formulation of the diagnostic conclusion and the choice of therapy [3, 4]. Secondly, the definition clearly states the key mechanisms of myocardial insufficiency: ventricular inotropic and lusitropic dysfunction. At the same time, the conjunction "or" actually confirms recognition of the existence of diastolic heart failure (DHF).

DHF has been known for a long time. Thinking and doubting researchers have a clear understanding that the so-called diastolic markers sometimes reflect the functional state of the myocardium and its reserve more accurately than systolic ones. It can be used more reliably than other hemodynamic parameters to assess the clinical status of a patient with CHF, the effectiveness of therapy, and the disease prognosis [5–13].

Important changes regarding the understanding of the pathogenesis of CHF marked the beginning of the millennium, and its first decade was called the decade of diastology [8, 14]. Even though numerous studies proved convincingly that the deterioration of the left ventricular (LV) filling pressure often precedes impairment of its systolic function and a number of factors affect (especially in the debut) mainly the diastolic function of the heart without changing the conditions of blood ejection [12, 15–20], modern classifications and approaches to CHF therapy are primarily based on the results of assessing the value of LV ejection fraction (EF) [1, 2, 21].

In recent years, DHF has been undeservedly forgotten and replaced by unequal terms based on determining the value of LVEF (for example, "CHF with preserved LVEF") [1, 2, 22–24]. At the same time, authors extensively use the term DHF since CHF based on myocardial insufficiency develops only through two mechanisms (systolic and / or diastolic dysfunction), and excluding one of them makes it possible to identify the underlying mechanism [22, 25–28].

The aim of this lecture is to consider views on the boundaries of the correct application of the term "diastolic heart failure".

LEFT VENTRICULAR SYSTOLIC AND DIASTOLIC DYSFUNCTION

LV systolic (from Greek systolé − contraction) dysfunction, which underlies classical systolic heart failure, is characterized by depression of its contractility. Traditionally, CHF is associated with depressed global LV systolic function, an indicator of which is a decrease in the LV ejection fraction (EF) [1, 2, 21, 29].

In general, the lower the EF value (the proportion of LV volume that is ejected into the aorta in the absence of abnormal blood flow into the systole), the worse the quality of life and the disease prognosis [1, 2, 21, 22]. However, the severity of clinical manifestations of heart failure in patients with LV systolic dysfunction does not always depend only on the value of EF. Patients with very low EF may have no symptoms or signs of heart failure, while patients with preserved LVEF can sometimes be 21severely decompensated [1, 22]. The discrepancy between the severity of clinical manifestations of heart failure and the level of depression of global LV contractility noted by many authors can be partly explained by the presence of valvular pathology, pericardial damage, changes in loading conditions (preload and afterload), rhythm and conduction disorders, or primary pathology of the right ventricle. Manifestation of CHF largely depends on the presence and type of LV diastolic dysfunction, as well as the effectiveness of hemodynamic and neurohumoral compensatory and adaptive mechanisms [16, 21, 30].

It should be remembered that the development of CHF is primarily associated with weakening of the pumping function of heart and the normal value of LVEF (systolic function). The exact calculation of LVEF largely depends on the choice of the imaging technique and the method of its calculation, as well as

on the skills of the operator; it is not a reliable indicator of intact cardiac output (LV pumping function). For instance, LVEF does not always correctly reflect the severity of global systolic dysfunction in primary and secondary valvular regurgitation, which is found in patients with CHF. In such cases, relative safety of EF can be combined with depression of LV pumping function (reverse blood flow leads to a decrease in stroke volume) and does not fully exclude systolic dysfunction, the signs of which can be detected using more informative methods and imaging techniques, in particular magnetic resonance imaging and two- and three-dimensional speckle tracking method [21, 31–36].

The term "heart failure with preserved (normal) LV systolic function" is usually used to refer to a situation with clinically significant heart failure and the absence of a pronounced decrease in the value of LVEF. However, in view of the above considerations regarding the accuracy of estimating the EF value, most often determined using two-dimensional echocardiography, when LV contractile dysfunction is ruled out, it is better to use the term "heart failure with preserved EF" rather than "heart failure with preserved (normal) LV systolic function" [1, 21].

Again, the classification of CHF phenotypes is based on the results of the LVEF assessment [2]. However, even in a state of relative rest, LVEF does not belong to rigid biological constants and can change in repeated examinations by one operator spontaneously or under the influence of treatment, regardless of the initial CHF phenotype, established by analyzing the value of this parameter, which obviously requires reclassification of heart failure, based on the changes in the value of the discussed parameter of global LV contractility (Figure) [1, 37].

LV diastolic (from the Greek diastolé − stretching) dysfunction is most often understood as a pathological condition when the ventricle cannot receive blood at low pressure and fill without a compensatory increase in atrial pressure (the average pressure in the pulmonary veins at rest does not normally exceed 12 mm Hg) due to impaired active relaxation of the myocardium and / or deterioration of its wall compliance [5, 38, 39]. The term "diastolic dysfunction" is used to refer to abnormalities in the active and / or passive mechanical properties of the whole ventricle (global diastolic dysfunction) or its segments (local diastolic dysfunction) in the diastole, regardless of whether EF is normal or reduced, and whether there are signs of heart failure [6, 19, 38, 40–42].

Thus, for example, if in a patient with hypertension without symptoms and signs of heart failure, an echocardiographic examination demonstrates normal EF and signs of LV diastolic dysfunction well described in modern guidelines [12, 13, 43–46], then this condition is referred to as asymptomatic diastolic dysfunction. [47, 48]. If such a patient with isolated LV diastolic dysfunction begins to experience shortness of breath, fatigue, and palpitations, a physical examination reveals signs of CHF, and laboratory tests show an elevated level of natriuretic peptides, then we can use the term DHF [6, 38, 40, 49–53].

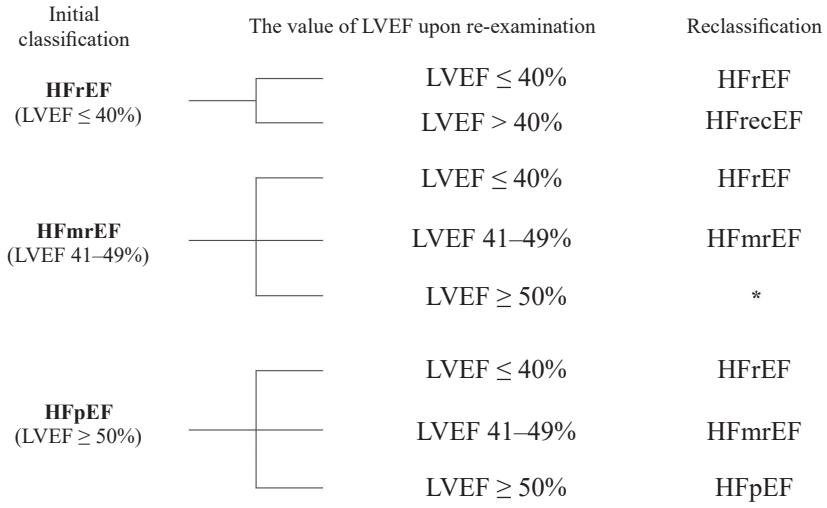

Figure. Classification and reclassification of CHF phenotypes [1]: HFrEF − CHF with reduced ejection fraction; HFmrEF − CHF with mildly reduced ejection fraction HFpEF − CHF with preserved ejection fraction; HFrecEF − CHF with recovered ejection fraction; * – CHF phenotype does not have a generally recognized designation

Thus, it is impossible to fully associate the concept of "diastolic dysfunction" with DHF: DHF always includes diastolic dysfunction, but the presence of the latter does not yet indicate the presence of the former [6, 47, 48]. In other words, the term "diastolic dysfunction" describes disturbances in the biomechanics of the heart in the diastole, while the term "DHF" is used to refer to the syndrome of heart failure in a patient with isolated diastolic dysfunction [40].

The existence of primary DHF is traditionally recognized. Wherein, it is believed that systolic dysfunction with unfailing regularity occurs in patients with diastolic dysfunction. The pathology of diastolic relaxation usually precedes systolic dysfunction, which appears later (the impaired filling pressure will sooner or later negatively affect the efficiency of the systole), but it is the appearance of systolic dysfunction, which accompanies diastolic disorders, that often manifests the clinical presentation of heart failure [6, 16].

Many patients with the classic systolic form of CHF also show signs of diastolic dysfunction [6, 16, 30]. Naturally, in this case, they do not talk about DHF. However, taking into account the independent clinical and prognostic value of LV inotropic and lusitropic dysfunction, the diagnostic conclusion should reflect "mixed" heart failure, for example, "CHF with reduced LVEF (38%) and restrictive LV diastolic dysfunction" [4, 42].

BOUNDARIES OF THE TERM APPLICATION

It is necessary to answer the question that many clinicians ask [26, 27]: is heart failure with preserved EF similar to DHF? Indeed, if LV systolic function is preserved in a patient with manifested CHF, it is logical to assume that LV diastolic dysfunction should underlie the development of such heart failure. However, despite the external validity, such a conclusion may be erroneous and is subject to reasonable criticism [28].

In our opinion, the known technical limitations of the possibility of an accurate non-invasive quantitative assessment of LV diastolic function [54, 55] are not a peremptory reason to completely dismiss the diagnosis of DHF in favor of the broader concept of "heart failure with preserved EF". To draw reasoned conclusion about DHF in a patient with objective signs of CHF and preserved LV EF, it is necessary to exclude all other cardiac and extra-cardiac causes that can lead to the development of heart failure with normal systolic function (in particular, cor pulmonale, pulmonary artery stenosis, primary tricuspid regurgitation), on the one hand. It is also necessary to confirm the presence of LV diastolic dysfunction (also in the so-called diastolic stress test), on the other hand [26, 28, 56].

The definition of LV diastolic dysfunction does not include patients with mitral stenosis, in whom impaired LV filling pressure and increased pressure in the left atrium are caused by a mechanical obstruction to blood flow in the left atrioventricular valve [6]. A similar judgment can be made regarding some other diseases, in which heart failure develops due to impaired LV filling pressure caused by external causes (constrictive pericarditis, pericardial effusion) [22, 26]. Since in this pathology relaxation of the LV myocardium is not impaired and / or myocardial stiffness is not increased, after timely correction (for example, valvotomy or effective removal of pericardial effusion), the left ventricle regains the ability to receive blood at low pressure and fill without a compensatory increase in pressure in the left atrium [22].

Thus, it is necessary to distinguish between heart failure that has developed following a primary impairment of active relaxation of the ventricular myocardium and / or deterioration of its wall compliance and heart failure in which disturbances of heart filling pressure, which underly it, are not the result of LV diastolic dysfunction.

CONCLUSION

The term "diastolic heart failure" can be used in clinical practice and cannot be replaced by an unequal diagnostic conclusion "CHF with preserved LVEF", since the latter is a broader concept referring to a full spectrum of cardiovascular diseases complicated by the development of CHF without depression of the global LV contractility and requiring the use of differentiated approaches to therapy. In addition, the results of repeated studies on the value of LVEF in many patients may require reclassification of this CHF phenotype, which is established by the analysis of the baseline value of global LV contractility. We join M.R. Zile [26] in the appeal to stop discriminating against the term "diastolic heart failure". However, it is necessary to clearly understand the boundaries of its correct application.

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Diagnosis of bacterial infection in patients with COVID-19: is it a simple task? (literature review)

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ABSTRACT

Diagnosing bacterial infection in patients with novel coronavirus infection (COVID-19) is not an easy task. Available data suggest that bacterial infection in patients with COVID-19 is rare and occurs in less than 10% of cases. At the same time, data of individual studies and systematic reviews indicate that more than 70% of patients with COVID-19 receive mainly empirical antimicrobial therapy with broad-spectrum antibiotics often before the diagnosis of COVID-19 has been verified. Therefore, this widespread empirical use of antibiotics is not supported by data on the need for their use.

The article discusses the literature data on the significance of commonly accepted methods for diagnosing bacterial infection, with an emphasis on laboratory presence / absence tests. In everyday practice, the likelihood of bacterial coinfection in patients with COVID-19 is assessed by clinical presentation of the disease and the results of standard laboratory tests and imaging methods. However, when viral respiratory infection develops, this approach does not always allow to diagnose bacterial coinfection with sufficient significance. This issue may be handled by available modern test systems, the use of a combination of signs or additional laboratory criteria (for example, procalcitonin), and the analysis of the overall clinical presentation by the doctor using knowledge about patient risk groups.

Keywords: COVID-19, bacterial infection, diagnosis

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Диагностика бактериальной инфекции у больных COVID-19: так ли все просто? (обзор литературы)

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РЕЗЮМЕ

Проблема диагностики бактериальной инфекции у больных новой коронавирусной инфекцией (НКИ) представляет не такую простую задачу, как выглядит на первый взгляд. Имеющиеся данные свидетельствуют, что бактериальная инфекция у больных COVID-19 встречается редко и составляет менее 10%. При этом данные отдельных исследований и систематических обзоров свидетельствуют, что более 70% пациентов

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с НКИ получали антибактериальную терапию, преимущественно препараты широкого спектра и часто эмпирически, нередко до получения подтверждения НКИ. Таким образом, это широко распространенное эмпирическое использование антибиотиков не подтверждается данными о необходимости их применения.

В статье обсуждаются литературные данные о значимости общепринятых методов диагностики бактериальной инфекции с акцентом на лабораторное подтверждение ее наличия/отсутствия. В повседневной практике сочетание клинического течения болезни и результатов стандартных лабораторных исследований, данных методов визуализации являются ведущими в оценке вероятности бактериальной коинфекции у пациентов с COVID-19. Однако в условиях развития тропной к респираторной системе вирусной инфекции такой подход не всегда позволяет с достаточной степенью достоверности диагностировать бактериальную коинфекцию. Помочь в этом могут имеющиеся современные тест-системы, использование комбинации признаков или дополнительных лабораторных критериев (например, прокальцитонина), а также анализ врачом общей клинической картины заболевания с использованием знаний о группах риска пациентов.

Ключевые слова: COVID-19, бактериальная инфекция, диагностика

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

2019 novel coronavirus infection (2019-nCoV), COVID-19, is an infectious disease that emerged in late 2019 and quickly spread around the world, having a significant impact on all sectors of healthcare. Since the beginning of the pandemic, doctors have faced various problems, the main of which were occasional lack of tests for prompt diagnosis and lack of commonly accepted treatment methods. This resulted in the situation when doctors frequently prescribed empirical antimicrobial therapy with broad-spectrum antibiotics to patients with lung lesions as part of COVID-19 treatment, despite the lack of evidence of bacterial coinfection. Empirical antibiotic therapy was often prescribed to critically ill patients when bacterial infection was suspected as the underlying cause. Viral lung disease may lead to bacterial superinfection, causing structural damage to lung tissue and weakening of host immunity. During previous influenza pandemics, the development of bacterial coinfection and superinfection was associated with increased mortality [1, 2]. Severe COVID-19 infection manifested itself by clinical and radiological symptoms and laboratory test results, mimicking those of bacterial pneumonia, so empirical antimicrobial therapy was a common practice early in the pandemic.

At the same time, available data indicate that bacterial infection in patients with COVID-19 is rare, occurring in less than 10% of cases [3–8]. However, the frequency of superinfection, especially in patients hospitalized in intensive care units, increased to 14% and, according to some data, up to 54% [4, 6, 9]. At the same time, data from individual studies and systematic reviews indicated that over 70% of patients with novel coronavirus infection received empirical antimicrobial therapy with broad-spectrum antibiotics often even before the diagnosis of COVID-19 infection has been verified [4, 6, 10–16]. Therefore, empirical antimicrobial therapy was prescribed although their necessity was not confirmed by the tests.

Given high frequency of antimicrobial therapy in COVID-19 (with such a low incidence of bacterial infection), it is yet to be understood what criteria are used by the doctor to make a decision to prescribe antibiotics to patients with this pathology. Several surveys of practicing physicians were conducted, which demonstrated the following results. A survey of 414 Italian doctors revealed that prescribing antibiotics to patients with novel coronavirus infection was due to accompanying comorbidities (bone marrow transplantation, presence of bronchiectasis), certain microbial isolation (positive test for pneumococcal urinary antigen or pneumococcal shedding), elevated levels of procalcitonin (PCT) (> 0.5 ng / ml), radiographic and ultrasound impressions of the thoracic cavity (presence of lobar consolidation), hospitalization in the intensive care unit (ICU), or mechanical ventilation [17].

In a survey of 166 physicians from 82 different hospitals in 23 countries, clinical presentation was recognized as the most important cause for initiating antimicrobial therapy, followed by laboratory markers of inflammation and X-ray findings. PCT was recognized as the most important factor among the laboratory inflammatory markers, followed by neutrophil and leukocyte counts and C-reactive protein (CRP) level [18].

According to Spanish researchers, fever (> 38 °C), cough, dyspnea, arthralgia, fatigue, anorexia and gastrointestinal symptoms, oxygen saturation < 90%, tachypnea or tachycardia, and wheezing on auscultation of the lungs were associated with prescribing antibiotics [15]. The decision to initiate antimicrobial therapy was made following elevated levels of conventional inflammatory markers, such as CRP (odds ratio (OR) 2.14, 95% confidence interval (CI) 1.91–2.41; $p < 0.05$), PCT (OR 1.73, 95% CI 1.28–2.35; $p < 0.05$), or leukocytosis (OR 1.18, 95% CI 1.01–1.38; $p < 0.05$), as well as increased levels of inflammatory markers associated with COVID-19, such as lactate dehydrogenase (OR 1.30, 95% CI 1.16–1.47; $p < 0.05$), interleukin 6 (OR 1.73, 95% CI 1.16–2.59; $p < 0.05$), or ferritin (OR 1.93, 95% CI 1.59–2.35; $p < 0.05$). The presence of any infiltrate on X-ray was also the reason for initiating antimicrobial therapy ($p < 0.05$).

A number of guidelines also set the criteria for antimicrobial therapy prescription. For instance, in order to detect fungal or bacterial pneumonia in patients with novel coronavirus infection, as well as to make a decision on the use of antibiotics, the recommendations by the National Institute for Health and Care Excellence (NICE) proposed to conduct the following [19]:

– complete blood count;

– thoracic imaging (radiography, computed tomography (CT) or ultrasound);

– bacterial culture tests of respiratory and blood samples (e.g. sputum sample or tracheal aspirate, blood culture);

– Pneumococcal and Legionella urinary antigen tests.

Russian guidelines recommend prescribing antimicrobial therapy only if there are convincing signs of bacterial infection: increased PCT (> 0.5 ng / ml), purulent sputum, leukocytosis (> 12×10^9 / l in the absence of previous use of glucocorticoids), and an increase in the proportion of band neutrophils (> 10%) [20].

Therefore, it is critical to conduct comparative studies to identify patients with COVID-19 who are candidates for empirical antimicrobial therapy, thereby reducing the widespread overuse of antibiotics.

The aim of our analysis was to summarize the results, risk factors, and methods of diagnosing bacterial infection in COVID-19 patients.

The review was prepared by searching relevant publications in PubMed, ResearchGate, and eLibrary databases, using the following keywords: COVID-19, SARS-COV2, diagnosis, and bacterial infection. The presented review is based on original research articles that discussed the evidence and significance of bacterial infections in COVID-19 patients and the conducted antimicrobial therapy. The review also included case studies, case series, observational studies, meta-analyses, and systematic reviews published from late December 2019 to May 2022.

RESULTS

It should be noted that, regardless of novel coronavirus infection, the diagnosis of a clinically suspected bacterial infection (especially pneumonia) is not always an easy task [21]. COVID-19 is a viral infection, but its clinical manifestations may be similar to those of bacterial pneumonia. Patients often have respiratory symptoms, including fever, cough, and dyspnea, and unilateral / bilateral changes in the lung tissue according to thoracic imaging. The most common radiological findings in these patients are ground-glass opacity, consolidation, and a combination of these two with a predominantly peripheral distribution. However, there are no specific radiological signs distinguishing between viral and bacterial pneumonia, especially atypical bacterial pneumonia. For example, one of the studies did not reveal significant differences in clinical symptoms and CT data in patients with novel coronavirus infection and a positive / negative urine test for pneumococcal infection [22]. This creates difficulties in differential diagnosis, especially before COVID-19 is verified.

A study conducted on patients with novel coronavirus infection compared the incidence of clinically established bacterial infection (bacterial pneumonia, urinary tract infections, ventilator-associated pneumonia (VAP), and bloodstream infections) with microbiological data [23]. In approximately 20% of COVID-19 hospitalizations, patients were diagnosed with bacterial pneumonia, and nearly all cases were community-acquired. In 9% of COVID-19 hospitalizations, patients were diagnosed

with community-acquired urinary tract infections. When microbiological results were used to detect bacterial infections, only about 7% of hospitalized COVID-19 patients had positive results for respiratory, blood, and urine cultures. Microbiological culture is a relatively insensitive method, especially during antimicrobial therapy [24].

Diagnosis of bacterial respiratory tract infection by bacterial culture test has two disadvantages: a significant waiting time (usually about 72 hours) for obtaining the result of a susceptibility test and low sensitivity of microbiological culture, which does not always make it easy to distinguish bacterial colonization from infection [25–28]. It is impossible to ignore the fact that the identification of bacterial pathogens does not explain the causal relationship. Some patients may be carrying large numbers of potentially pathogenic bacteria either in their respiratory tract or in the endotracheal site during intubation without developing any clinically significant bacterial infection [29]. For instance, according to the study by S. Khurana et al. (2021), only 60% of samples obtained from patients with novel coronavirus infection and clinical signs of bacterial infection tested positive for bacterial culture, including samples classified as contaminants [28].

According to C. Huang et al. (2020), 98% of patients with COVID-19 had bilateral lung lesions on chest X-ray, and only 28% of patients provided sputum for Gram stain or culture [30]. Concerns about performing aerosol-generating procedures (e.g. bronchoalveolar lavage) further limit the ability to obtain satisfactory sputum samples for bacterial culture tests and other microbiological tests [31–32]. It should also be taken into account that in a number of countries, guidelines for management of patients with community-acquired pneumonia do not recommend routine staining and culture of sputum in patients with non-severe chronic obstructive pulmonary disease (COPD) or in patients without the risk of multiple drug resistance due to low sensitivity of these tests [32]. Urine sampling for Legionella and pneumococcal antigen is not common in Russia due to its high cost, while microbiological testing of respiratory samples is routinely performed in patients hospitalized for COVID-19. Consequently, the possibility to diagnose bacterial infection in outpatients with novel coronavirus infection is significantly limited.

Standard laboratory methods are too slow to make initial decisions on prescription of antibiotics, requiring the use of rapid point-of-care testing. This technology which is promoted as a solution to future rational use of antimicrobials is now available and can provide comprehensive panel results for respiratory viruses, including SARS-CoV-2, within 45 minutes. Recently developed rapid tests can improve both the speed and sensitivity of examination [26, 33, 34]. Before the emergence of novel coronavirus infection, the US Food and Drug Administration approved the use of many multiplex PCR panels to aid in early diagnosis of possible respiratory pathogens, including Luminex xTAG RVP v1 (Luminex Corporation), Luminex xTAG RVP Fast (Luminex Corporation), FilmArray respiratory panels (BioFire Diagnostics), BioFire FilmArray Pneumonia / Pneumonia plus (BioFire PN/PNplus; BioFire Diagnostics, LLC, Salt Lake City, UT), eSensor RVP (GenMark Diagnostics), Verigene Respiratory Pathogens Flex Test (Luminex Corporation), Luminex NxTAG Respiratory Pathogen Panel (Luminex Corporation), and ePlex Respiratory Pathogen Panel (GenMark Diagnostics) [35].

During the COVID-19 pandemic, SARS-CoV-2 was quickly incorporated into pre-existing syndromic multiplex panels, such as QIAstatDx Respiratory 2019-nCoV (Qiagen, Netherlands) and BioFire FilmArray RP-2.1 (BioFire FilmArray Respiratory Panel-2 plus SARS-CoV- 2; bioMerieux, France) [35]. The panels are molecular multiplex PCR tests that increase the sensitivity of detecting causative agents of pneumonia and significantly reduce the risk of misdiagnosis of coinfection during the COVID-19 pandemic. Some studies have already discussed using some of them to diagnose the bacterial coinfection in patients with novel coronavirus infection [9, 36, 37]. According to Z. Dhesi et al. (2020), the FilmArray Pneumonia Panel revealed bacterial infection in 54% of patients, while the microbiological culture data detected it in only 28.2% of cases [9]. In another study, early bacterial coinfection in patients with acute respiratory distress syndrome was reported in 13 (27.7%) patients with COVID-19: in 12 people following a PCR test and only in one individual following conventional bacterial culture test [38].

A systematic review and meta-analysis involving the results of seven studies (558 patients with novel coronavirus infection admitted to the ICU) demonstrated that the FilmArray Pneumonia panel detected bacterial infection in 33% of cases (95% CI 0.25–0.41, I2= 32%), while bacterial culture test revealed it in only 18% of patients (95% CI 0.02–0.45; I2=93%) [26]. Similar data on greater information value of multiplex panels were reported by other authors [37]. In another study, a rapid PCR test panel (ABI 7500 Real-Time

PCR System, Applied Biosystems, USA) detected 31 more respiratory pathogens, including *P. aeruginosa, E. coli, M. catarrhalis, H. influenzae*, *S. pneumonia*, and *S. pyogenes*, than conventional bacterial culture tests [39].

The need to obtain a high-quality sputum sample limits the use of such panels outside the ICU, which is not always possible in non-intubated patients, e.g. due to the absence of cough. In addition, a number of authors reported complicated interpretation of the test results, limitations of the panel in the detection of gram-negative bacteria, and sometimes overdiagnosis of MRSA [36], albeit such panels could help resolve the issue of distinguishing infection from colonization via semi-quantitative results [26]. Although the prospects of these tools for diagnosing infectious diseases are great, their superiority over conventional mainstay approaches, such as bacterial culture tests, has not yet been unequivocally confirmed.

Another method aimed directly at the detection of pathogens is microbial metagenomic sequencing (MS). This is a rapidly developing technology that allows to identify pathogen and microbiome information simultaneously within 24 hours [5, 40]. Based on previous studies, it can be concluded that MS has higher detection sensitivity than conventional microbiological tests, which makes this method more advantageous in modern microbial surveillance [5, 41]. A microbiome analysis is increasingly used in clinical microbiology laboratories to identify rare and hard-to-detect pathogens and coinfections directly from clinical samples [40]. Characterization of the respiratory microbiome was performed in various respiratory diseases.

In VAP, sequencing of 16S rRNA amplicons expanded microbiological diagnosis by complementing standard culture and demonstrated its potential use as a prognostic marker, since the composition of the microbiome during intubation can predict subsequent development of VAP [40]. MS of COVID-19 respiratory samples demonstrated minimal differences in the microbiome between patients with different prognosis and patients with different duration of mechanical ventilation. At the same time, bronchoalveolar lavage samples are comparable in the information value with samples obtained following endotracheal aspiration [5, 40]. In a small Danish study (34 patients), potential pathogens were detected in 7 patients (21%) by the bacterial culture test, in 4 patients (12%) by the microbiome analysis, and in only 1 patient (3%) by the respiratory panel. The authors considered that there was a reasonable agreement between the results of the bacterial culture test and the microbiome analysis, as 6 / 7 (85%) of the cultured microorganisms in the aspirates were identified during the microbiome analysis. When combining the results of the microbiome analysis and conventional bacterial culture tests in endotracheal aspirate samples, the prevalence of bacterial / fungal coinfections increased from 21 to 33% [40].

The increased sensitivity of multiplex panels and 16S metagenomic analysis for the detection of pneumonia-inducing pathogens, compared with bacterial culture tests, was demonstrated in the European multicenter study BioFire PNplus, Curetis Unyvero [34]. The scope of application of the microbiome analysis in respiratory specimens in the clinical setting is yet to be determined, but its routine use requires reduced processing time and cost.

PREDICTORS (BIOMARKERS) OF BACTERIAL COINFECTION AND SECONDARY INFECTIONS

Studying the predictive capability of various clinical and laboratory tests as predictors of bacterial coinfection and secondary infections is relevant. PCT is recognized as the most promising indirect test in terms of diagnosing bacterial infection in patients with novel coronavirus infection [6, 20, 42, 43]. PCT, a precursor of the hormone calcitonin, is stimulated by IL-6, tumor necrosis factor, and cytokines associated with bacterial infection and is inhibited by interferon gamma, which is associated with viral infections [44]. Expression of PCT is elevated in the epithelial layer of cells when infected with bacterial infection. A landmark article published in 1993 reported on the ability of PCT to distinguish between bacterial and viral infections [45]. A number of studies showed that PCT surpasses CRP in distinguishing between bacterial and viral infections [46], but the role of PCT measurement in antimicrobial management is controversial. PCT testing is approved by the US Food and Drug Administration for the treatment of sepsis and respiratory tract infections; but in the UK, current guidelines of the National Institute for Health and Care Excellence (NICE) do not include PCT testing due to insufficient evidence [47].

Many studies showed that antimicrobial therapy with the control of PCT levels yields good results in patients with acute respiratory disease, exacerbation of COPD, and sepsis [24, 44, 48, 49]. Numerous studies demonstrated a higher level of PCT in patients with

COVID-19 and bacterial coinfection compared with patients with COVID-19 without signs of bacterial infection, as well as changes in PCT after the initiated antimicrobial therapy [24, 50–52].

During the first wave of the COVID-19 pandemic, there was widespread use of PCT level detection in NHS hospitals (UK). The number of hospitals using PCT detection for emergency / acute hospital admissions increased from 17 (11%) to 74 / 146 (50.7%), and its use in ICUs increased from 70 (47.6%) to 124 / 147 (84.4%). This increase occurred predominantly in March and April 2020, before the release of the NICE guidelines. Approximately half of the hospitals used PCT measurement as the only test to decide on discontinuation of antimicrobial therapy, and half of the hospitals used repeated measurements [47].

In their study, M. van Berkel et al. (2020) investigated PCT and CRP levels as prognostic markers of possible bacterial coinfection in COVID-19 patients in the ICU. It was noted that CRP levels tend to increase, while PCT is often low in patients with novel coronavirus infection at admission [53]. Other authors presented similar data on PCT as a prognostic marker in COVID-19 [24].

In the study by G.P. Drewett et al. (2021), changes in serum PCT levels were associated both with the initiation of antimicrobial therapy in patients with novel coronavirus infection and with the transition from intravenous to oral drug delivery [54]. All patients with high PCT levels (> 0.5 ng / ml) received antibiotics in hospital, while 20% of patients with moderate PCT levels (0.07– 0.5 ng / ml) and 40% of patients with low PCT levels (< 0.07 ng / ml) did not receive any antimicrobial therapy. These results highlight the potential utility of PCT testing. Similar data on lower frequency of prescribing antibiotics (21%) in the absence of an increase in PCT level (< 0.25 ng / ml) were obtained by the authors of another study [52].

The most appropriate threshold for PCT has not been determined yet [19]. It was indicated that a PCT level of more than 0.5 ng / ml could be used to confirm bacterial infection, while a level of < 0.25 ng / ml was not associated with bacterial infection [43]. According to M. van Berkel et al. (2020), the negative predictive value in patients with PCT levels < 0.25 μg / l was 81%, whereas PCT >1.00 μg / l had a positive predictive value of 93% for the development of bacterial infection [53]. Data from another study suggested that the use of PCT levels as a marker for reducing the use of antibiotics actually decreased the duration of their use by two days in patients with novel coronavirus infection [55].

However, not all studies confirmed the diagnostic value of PCT [8, 44, 56, 57]. In a retrospective analysis of the data from 60 patients with COVID-19, no significant difference was found between peak PCT levels in patients with positive and negative bacterial culture tests [58]. Another case–control study demonstrated that no difference was observed between PCT levels in COVID-19 patients with and without bacterial coinfection (p = 0.883) [56]. Besides, an increase in PCT levels can be detected in patients with novel coronavirus infection without bacterial coinfections, which would serve as a basis for prescribing antibiotics. For instance, in one study, microbiologically confirmed bacterial infection was present in only 12% of patients with a PCT level of > 0.5 ng / ml [32].

Therefore, the significance of PCT in detecting bacterial coinfection is not so clear, and further research is needed to develop an accurate predictive model or a method for diagnosing coinfection in patients with novel coronavirus infection. The NICE guidelines (UK) state that there is not enough evidence for introducing a PCT level examination for making decisions on prescription of antibiotics [19]. In October 2020, the study on the significance of PCT levels in antibiotic use in hospitalized patients with COVID-19 (PEACH), commissioned and funded by the National Institute of Health Research (NIHR), started [59].

Therefore, the significance of an elevated PCT level in confirming the presence of bacterial infection in patients with novel coronavirus infection is not undoubtful: its increase may represent bacterial infection or immune dysregulation, or actually be a marker of the disease severity. However, low or normal PCT levels may be useful to avoid prescription or early discontinuation of empirical antimicrobial therapy in non-critically ill patients. Besides, serial measurements of PCT provide the insight into inflammatory changes in the patient, where a secondary increase should make the physician suspect a bacterial superinfection [60]. Finally, PCT levels can be controlled after the initiation of antimicrobial therapy to reduce the duration of treatment [60].

Some authors emphasized the diagnostic value of leukocytosis (especially neutrophilia) in patients with COVID-19 in relation to bacterial infection [7, 8, 51, 61–64]. The results of another study indicated that the

level of leukocytes less than 8.2×10^9 cells / l allowed to exclude bacterial infection in 46% of patients with COVID-19 [25], while other authors recommended focusing on a level $\geq 10 \times 10^9$ / l [65].

Regarding the level of CRP, there are recommendations that its high values in patients with novel coronavirus infection do not necessarily imply the presence of bacterial infection; however, its low level characterizes a low probability of bacterial coinfection [19, 53, 66]. In a study by German researchers, patients with COVID-19 and confirmed coinfections had higher levels of CRP and PCT than patients without infection [67]. In bloodstream infections, the increase in CRP and PCT levels was more pronounced than in respiratory infections.

The study by P. Hedberg et al. (2022) showed that the predictors of bacterial coinfection in patients with COVID-19 were CRP levels of ≥ 50 mg / l, CRP levels of ≥ 150 mg / l, leukocyte count of over 12.0×10^9 cells / l, PCT levels of ≥ 2.00, and the neutrophil-to-lymphocyte ratio exceeding 20.0 [68].

H. Chen et al. (2021) considered prescribing antibiotics only if the infiltrate was visible on chest X-ray and the leukocyte count was $\geq 10 \times 10^9$ / l, or in severe illness requiring intensive care in the ICU. If the CRP level was < 60 mg / l or the PCT level was < 0.5 ng / ml, they recommended to refrain from prescribing antibiotics [65]. According to the authors, only 4 out of 114 patients would qualify for antibiotics during a 14-day period.

It should be noted once again that serological markers of inflammation usually associated with bacterial infection, such as elevated PCT and CRP levels, may appear in patients with novel coronavirus infection without bacterial coinfection [69, 70].

RISK FACTORS FOR BACTERIAL INFECTION

It is often difficult to determine which patients should be given antibiotics and which patients may not benefit from them. The possibility of identifying a probable bacterial pathogen in a large number of admitted patients with novel coronavirus infection is significantly limited. Therefore, it is necessary to understand and identify risk factors for the development of bacterial infections in hospitalized patients with COVID-19 and reveal markers of a bacterial coinfection in order to form clearer indications for antimicrobial therapy. All of these would contribute to rational antibiotic use aimed at improving the quality and safety of their application.

Risk factors for the development of bacterial infection include age over 60 years, prolonged hospital stay, need for mechanical ventilation, stay in the ICU (severe course of COVID-19), chronic bacterial infections in the past medical history (primarily those of the respiratory tract), administration of steroids and / or immunosuppressive therapy prior to and / or during COVID-19 therapy, chronic renal failure with a need for hemodialysis, and immunodeficiency (e.g. chemotherapy for cancer; bone marrow or organ transplantation; primary immunodeficiency; poorly controlled HIV or AIDS) [3, 4, 16, 20, 29, 32, 50, 51, 66, 67, 71–74]. It is also impossible to ignore the increased risk of catheter-associated bacterial infection in patients with a severe course of novel coronavirus infection following even short-term placement of a central catheter [27, 75].

CONCLUSION

Therefore, the problem of diagnosing bacterial infection in patients with COVID-19 appears quite complicated. In routine practice, the combination of the clinical course of the disease with the results of standard laboratory tests and data provided by imaging methods are foremost in assessing the likelihood of bacterial coinfection in patients with novel coronavirus infection. However, this approach does not always allow to diagnose bacterial coinfection with a sufficient degree of certainty when a patient develops viral respiratory infection. Substantial assistance in this matter could be provided by available modern test systems along with a combination of symptoms and additional laboratory criteria (e.g. PCT), supplemented with the analysis of the overall clinical presentation of the disease performed by a physician with the knowledge of the patient risk group. Furthermore, the data on extremely rare incidence of bacterial infection in outpatients and its rare incidence in patients in the first 5–10 days of hospitalization are very helpful in this regard.

If the doctor doubts the presence of bacterial coinfection, empirical antimicrobial therapy is possible upon admission of the patient to the hospital (in the first 24–48 hours). However, after receiving laboratory test results, antimicrobial therapy should be reviewed and immediately discontinued if there are no criteria for its prescription. Consequently, young patients and patients without concomitant pathologies who are prescribed antibiotics for dry cough, fever, flu-like symptoms, interstitial infiltrates, or elevated CRP levels are likely to receive antimicrobial therapy

without indications. Therefore, in the absence of alternative data indicating the need for its use, antimicrobial therapy should be suspended.

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Foresight in the diagnosis of trematodiasis: innovations versus routine methods

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ABSTRACT

Aim. To analyze modern methods for the diagnosis of trematodiasis in experimental and epidemiological studies.

Trematodiasis is a group of common parasitic diseases that are a socially sensitive health problem worldwide. According to World Health Organization, more than 250 million people are affected by trematode infections globally. The most common types of human trematode infections are diseases caused by *Schistosoma, Fasciola, Clonorchis*, and *Opisthorchis* pathogens. Diagnosis of trematodiasis is often multistage and includes identification of disease symptoms, analysis of medical history, and use of various laboratory tests. Clinical presentation of parasitic infections often varies, making a definitive diagnosis difficult. Various tools are used to diagnose trematode infections: epidemiological criteria, laboratory tests (complete blood count and blood biochemistry, serological methods), instrumental methods (abdominal X-ray and ultrasound), and parasitological techniques, which often have insufficient sensitivity and specificity. Therefore, development of modern and effective non-invasive methods for detection of trematode infections with high sensitivity and specificity, including screening in endemic regions, is relevant.

The present review analyzes the results of 90 clinical trials and experimental studies on the diagnosis of trematode infections using the PubMed search engine and the eLibrary database. The review analyzes original articles published from January 1, 2015 to December 31, 2021.

Most studies confirm that the absence of a standard diagnostic approach highlights obvious convenience of utilizing a combined approach to reliable diagnosis of trematodiasis. An adequate combination of different diagnostic tests makes it possible to diagnose the disease correctly, devise a correct treatment and follow-up strategy, and organize preventive measures.

Keywords: trematode, opisthorchiasis, clonorchiasis, duodenal probe, microscopic helminth detection, immunoassay, molecular diagnosis, molecular genetic diagnosis, endoscopic examination, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound examination (US)

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Форсайт диагностики трематодозов: инновации против рутинных методов

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РЕЗЮМЕ

Цель данного обзора литературы – провести анализ современных методов диагностики трематодозов в экспериментальных и эпидемиологических исследованиях.

Трематодозы – распространенные паразитарные заболевания, являющиеся важной проблемой общемирового здравоохранения. По данным Всемирной организации здравоохранения, трематодозами поражено более 250 млн человек. Наиболее распространенными видами трематодозов человека являются заболевания, вызванные возбудителями *Schistosoma, Fasciola, Clonorchis* и *Opisthorchis*. Диагностика трематодозов часто бывает комбинированной и многоступенчатой – выявление симптомов заболевания, сбор эпидемиологического анамнеза и использование различных лабораторных исследований. Клиническая картина паразитарных инвазий часто варьирует, что затрудняет окончательный диагноз. Для диагностики трематодозов используют различные диагностические инструменты: эпидемиологические критерии, методы лабораторной диагностики (общий и биохимический анализ крови, серологические методы), инструментальные методы (рентгенологические и ультразвуковые исследования органов брюшной полости), паразитологические методы, нередко обладающие недостаточной чувствительностью и специфичностью. В этой связи актуальна разработка современных и эффективных неинвазивных способов детекции трематодозов, в том числе для скрининговой диагностики в эндемичных регионах.

В работе проведен анализ 90 научных публикаций результатов клинических и экспериментальных исследований в области диагностики трематодозов с использованием электронно-поисковой системы PubMed и научной электронной библиотеки Elibrary. В обзоре представлены оригинальные статьи, опубликованные с 1 января 2015 г. по 31 декабря 2021 г.

Большинство исследований подтверждает, что отсутствие стандартного диагностического подхода подчеркивает очевидное удобство применения комбинированного подхода для получения достоверного диагноза трематодоза. Адекватное сочетание различных диагностических тестов позволяет правильно диагностировать заболевание, составить правильный план лечения и последующего наблюдения, организовать меры профилактики.

Ключевые слова: трематодоз, описторхоз, клонорхоз, дуоденальное зондирование, гельминтоовоскопия, иммуноферментный анализ, молекулярно-генетическая диагностика, эндоскопическое исследование, компьютерная томография (КТ), магнитно-резонансная томография (МРТ), ультразвуковое исследование (УЗИ)

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с содержанием настоящей статьи.

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INTRODUCTION

Trematodiasis is a group of common parasitic diseases that are a socially sensitive health problem worldwide. According to the World Health Organization (WHO), it affects more than 250 million people in many countries on five continents [1–5]. The most common types of human trematode infection are diseases caused by *Schistosoma, Fasciola, Clonorchis,* and *Opisthorchis*. People acquire trematodes while

swimming via skin and mucous membranes, by accidental ingestion of water containing parasite cysts, and by consumption of inadequately processed or raw fish.

Helminth invasion of the biliary tract is an important medical problem, especially in tropical and subtropical endemic areas [6]. Many endemic regions (China, Philippines, Cambodia, Laos) have launched national programs to achieve control of invasions [7]. Trematodiasis control worldwide is aimed at reducing the risk of infection. For this purpose, mass prophylactic anthelmintic treatment is offered in endemic areas. All this is due to difficult diagnosis of these diseases, which is often combined and multistage and includes identifying symptoms of the disease, taking a biological history, and using various laboratory tests. The clinical presentation of parasitic infections often varies, making a definitive diagnosis difficult. Various diagnostic tools are used to diagnose trematodes: epidemiological criteria, laboratory diagnostic methods (complete blood count and blood biochemistry, serological tests), instrumental methods (abdominal X-ray and ultrasound), parasitological techniques [8]. Specific diagnostic methods are detection of eggs in feces, urine, and / or duodenal contents [6, 9]. However, it is recognized that parasitological methods show low sensitivity in detecting mild infestations; consequently, there remains a need to develop more sensitive and specific diagnostic tools to monitor the prevalence of parasite invasion [9–12].

The aim of this literature review was to analyze modern methods for the diagnosis of trematodiasis in experimental and epidemiological studies.

METHODS

The present review analyzes the results of clinical trials and experimental studies on the diagnosis of trematode infections using the PubMed search engine and the eLibrary database. The review includes original articles published from January 1, 2015 to December 31, 2021. Primary search for publications on the diagnosis of trematodiasis was carried out. The keywords used for the search were “trematodiasis”, “opisthorchiasis”, “clonorchiasis”, “duodenal sounding”, “helminthovoscopy”, “enzyme-linked immunosorbent assay”, “metabolomics”, “proteomics”, “molecular genetic diagnosis”, “PCR”, “endoscopic examination”, “CT”, “MRI”, “ultrasound”, “X-ray”. Approximately 350 publications in Russian and over 1,500 publications in English were identified.

At the first stage, we selected articles the title of which mentioned methods for diagnosing trematodiasis; at the same time, we excluded reviews and articles duplicating information. At the second stage, we analyzed abstracts of the publications and excluded works in which routine methods and stool microscopy were used as the only research methods. At the third stage, we selected articles with access to the full text; as a result, a detailed analysis of 90 publications containing data on original modern research in the field of diagnosing trematodiasis was carried out.

MODERN MICROSCOPY TECHNOLOGIES

The results of the analysis indicate wide geographical coverage of the studies: most of them were performed in the endemic regions of Southeast Asia; however, some studies were carried out in Europe, USA, and Africa [12–15]. To confirm the diagnosis of most trematode invasions, microscopy of patient stool samples is used, since the method is simple and affordable; however, it only detects invasion of medium and high intensity [12, 16]. The sedimentation method has greater sensitivity and versatility at low invasion intensity [15, 17, 18]. Microscopy of stool, urine or duodenal samples requires a laboratory with a light microscope and qualified experienced personnel, which is not always available in endemic areas.

Some researchers propose a solution to the above problems with modern compact microscopes compatible with smartphones. So, in the study [19, 20], a comparison of a standard microscope and two mobile microscopes – Foldscope and CellScope – was carried out for the diagnosis of *S. haematobium*. Sensitivity was 55.9 and 69.6%, specificity reached 93.3 and 100%, respectively. Given possible technical improvement (increased sensitivity, increased fields of view), these devices can become pilot technology for the production of portable diagnostic microscopes.

At the same time, for the differential diagnosis of schistosomiasis, a rectal biopsy can be used, which makes it possible to achieve high sensitivity of a microscopic examination in comparison with standard serological tests [21].

ENZYME IMMUNOASSAYS

Enzyme immunoassay is a common and frequently used method in parasitology. The most common technique is enzyme-linked immunosorbent assay (ELISA). This method is based on detection of specific antibodies to present parasites secreted by the

body or parasite antigens themselves determined in venous blood or urine. This method has advantages over stool microscopy, since parasite antibodies are detected even in case of slight invasion. A study investigating seroprevalence and the relationship between the number of *O. viverrini* eggs in infested individuals and the specific antibody response showed that ELISA (based on total IgG and IgG4 antibodies to *O. viverrini*) has higher sensitivity in the analysis (98.4 and 89.8%, respectively) than the modified formalin-ethyl acetate technique (70.3%). The study also noted a positive correlation between the number of *O. viverrini* eggs in one gram of stool and IgG antibody levels [7].

However, with enzyme immunoassays, both false positive and false negative results are possible: the former appear against the background of a previous invasion, the latter – in severe immunodeficiency [22, 23]. An important disadvantage of ELISA is also the presence of cross-reactions due to antigenic similarities of various parasites. In this regard, an important task is to increase the sensitivity and specificity of the method.

Thus, to confirm the invasion by *S. japonicum*, the diagnostic potential of cathepsin B was studied (SjCatB). The results showed high sensitivity (86.7%) and specificity (96.7%), which indicates the promising diagnostic potential of this marker [24, 25].

The results of the literature review indicated that extravesicles [24, 26], P-selectin [27], soluble egg antigens [28–31], and serum immunoglobulins [32] were used to develop diagnostic tools for schistosomiasis. As part of the experimental work, specific proteins were studied in animals [33, 34], e.g. saposin-like proteins (SjSAP4 + Sj23-LHD (large hydrophilic domain)) provided the best diagnostic result with the sensitivity of 87.04% and specificity of 96.67% [24, 35–37]. A study of the inflammatory marker YKL-40 (chitinase-3 like-protein-1, also called human cartilage glycoprotein 39) in preschool children showed that it could be a potential biomarker of *S. haematobium* [38]. Serum carbonic anhydrase 1 (CA1) was proposed as another line of *S. mansoni* diagnosis [39].

To simplify screening, the point-of-care circulating cathodic antigen assay is utilized (POC-CCA), in particular, kits for detecting *S. mansoni* are used [23, 34, 40–44].

A work evaluating the use of three functionally active recombinant forms of the main secreted cathepsins (rFhCL1, rFhCL2, rFhCL3) and cathepsin B, rFhCB3 as antigens in indirect ELISA for serological diagnosis of *F. hepatica* infection in experimental and natural conditions showed that the level of antibodies to all three cathepsins L remained high during chronic invasion, but quickly decreased after drug treatment, which can be used to assess the effectiveness of deworming [45, 46].

Currently, the availability of omics studies makes it possible to study the parasite proteome and use these data to create new diagnostic kits. To improve the sensitivity, specificity, or field applicability of diagnostic kits, a search was performed for serum and urine biomarkers to determine *S. haematobium* invasion [22]. Another study evaluated the possibility of using milk instead of blood serum for early diagnosis of fascioliasis in dairy goats [47].

In the study on methods for diagnosing *F. hepatica* using ELISA, a group of researchers proposed to use a multiepitope construct of three protein epitopes cathepsin L1, a saposin-like protein 2 (SAP-2), and a 16.5-kDa tegument-associated protein (FhTP16.5), showing its high antigenicity and specificity [48]. At the same time, cathepsin L1 as a separate marker can act as a suitable antigen as well [49–52]. Some researchers studied fractionated parasite cells by conducting ELISA of proteins of different mass, rapidly yielding a reproducible method for obtaining antigens with acceptable sensitivity and specificity [53].

Monoclonal antibodies (MoAb) against recombinant *F. gigantica* glutathione peroxidase (rFgGPx) can be used for immunodiagnosis of both early and advanced fascioliasis in animals and humans [54, 55], and recombinant adenylate kinase 3 is proposed as a marker for the serodiagnosis of clonorchiasis [56]. Determining the cathepsin level in *O. viverrini* invasion also shows good results (sensitivity and specificity of 62.1 and 84.1%, respectively), so it can become an alternative immunodiagnostic tool for human opisthorchiasis in endemic areas [57, 58]. The use of monoclonal antibodies in *O. viverrini* invasion proved to be promising due to high diagnostic accuracy with the possibility of using urine samples instead of stool samples. Comparison of the concentrations of monoclonal antibodies in urine samples and stool samples showed a positive relationship between antibody concentrations and the number of eggs in one gram of feces, as well as high specificity [59–61].

In another study that used gold nanoparticles, a more than 3-fold rise in the sensitivity of the method for

detecting *O. viverrini* antigens was achieved compared to the classical method, increasing the sensitivity to 93.8 vs. 91.3%, respectively. The innovation allowed to reduce the number of reaction steps, which leads to a decrease in the waiting time for the results without a decrease in the quality of the analysis [62].

MOLECULAR GENETIC METHODS

Another method that has become widespread in the diagnosis of parasitological diseases is the polymerase chain reaction (PCR), based on detection of parasite antigens in various samples – blood serum, urine, stool. When comparing modern invasion detection methods, PCR surpasses standard microscopy of stool and urine samples and most often shows fairly high sensitivity and specificity [41, 46, 63–66]. When compared with standard methods of parasitological diagnosis, PCR shows a significant increase in the disease prevalence [67]. Thus, in a study, PCR revealed 13–15% more cases of invasion compared to microscopy [68]. With the improvement of methods for extracting DNA from samples, the sensitivity of the method can reach 100%. For example, when using the method in the experiment of mechanical disruption of parasite eggs by bead beating, a 100% positive result in the diagnosis of *S. haematobium* was achieved compared to 85% using the standard extraction procedure [69].

Along with PCR, recombinase polymerase amplification (RPA) is carried out, which allows to increase sensitivity from 66 to 87%, respectively, with 100% specificity of both methods. In fascioliasis, RPA helps to detect 47% of infections not detected by microscopy [70, 71]. The real-time RPA for diagnosing urogenital schistosomiasis targeting the *S. haematobium* Dra 1 sequence had clinical sensitivity and specificity of 98.4 and 100%, respectively, compared to the real-time PCR assay also targeting the Dra 1 sequence [72].

Another modification of PCR is loop-mediated isothermal amplification (LAMP) – a DNA amplification technique in one tube, which allows for molecular diagnosis to be carried out much cheaper and faster than PCR. A study on diagnosing *S. haematobium* invasion using LAMP showed similar sensitivity and specificity compared to PCR (100%). For *S. mansoni*, sensitivity was higher for LAMP amplification (100%) than for PCR (99%) [73]. The same method was used to study stool specimens from patients with suspected *C. sinensis* infection, achieving high sensitivity and specificity [74].

In another study, traces of *F. hepatica* infection were also determined in comparison with the standard PCR method [75]. This disease is characterized by difficult detection, often despite the symptoms, standard parasitological methods do not detect the presence of eggs. In this article, LAMP analysis showed a positive reaction even if there was only one helminth egg in the sample. In addition, visualization of the result is also quite simple – a color change in the solution is observed under ultraviolet light. Similar results were presented in a study comparing classical parasitological methods (direct wet preparation and concentration method), PCR, and LAMP for the diagnosis of *F. hepatica* in sheep fecal samples [18]. Also, the LAMP analysis allows to avoid cross-reactions with other species. In a study evaluating LAMP for the detection and monitoring of *S. mansoni* transmission foci in the intermediate host (snail of the genus *Biomphalaria)*, the LAMP method was shown to be three times more efficient than parasitological testing and more convenient for field use than other molecular methods [76].

Quite a lot of studies are being carried out to search for new targets for the diagnosis and differentiation of various invasions and the search for new antigens and their combinations [10, 15, 18, 24, 33, 65, 77–84]. As new markers, saposin-like protein [37], miRNAs isolated from serum extravesicles [85, 86], and cathepsin L3 are used in schistosomiasis [87], 27-kDa purified parasite antigen is used in fascioliasis [14], and intergenic spacer region of *F. hepatica* DNA [17] and schistosomule antigen are used in *S. mansoni* invasion [88].

The sensitivity of PCR-based methods depends on the number of copies of the gene, the number of parasite eggs, the quality of DNA, and PCR inhibitors in the sample. There are studies on improving PCR methods by influencing the *ITS2*, *cox1*, and *cyb* genes [82].

With the ever-improving capabilities of sequencing and bioinformatics tools for analyzing sequences and other highly repetitive genomic elements, real-time PCR as a diagnostic tool is becoming more common. A cluster analysis of the *S. japonicum* genome was carried out, on the basis of which a primer – probe set was developed. The resulting real-time PCR test was shown to reliably detect as little as 200 μg of *S. japonicum* genomic DNA, and when testing tiny stool samples containing a single helminth egg, the designed primer – probe set detected DNA in all samples [89]. Multiplex real-time PCR for the diagnosis of *S. haematobium* and *S. mansoni* was analyzed on

serum samples [13]. Sensitivity and specificity values based on multiplex real-time PCR for diagnosing *S. haematobium* and *S. mansoni* were calculated in the range of 87.9–100%.

Despite the advances in the use of molecular genetic methods in the diagnosis of trematodiasis, the study of new potential targets causes difficulties associated with insufficient sensitivity and cross-reactions. Thus, in the study of real-time PCR based on SjR2 and SjCHGSC19, it was shown that these markers can be used to diagnose *S. mekongi* infections in human blood serum, but the tests had low sensitivity and cross-reactions with samples positive for *S.mansoni* or *S. haematobium* [16].

IMAGING METHODS

Considerable attention is paid to imaging technologies in the diagnosis of helminthiases [90]. One study examined cases of appendicitis associated with schistosomiasis [91]. As a result, the features of computed tomography (CT) data were determined, which can be used as criteria for early detection of *S. japonicum* – large diameter, calcifications in the appendix wall together with the sigmoid colon and calcifications in the caecum, signs of perforation or abscess. Another study in Africa found that in patients diagnosed with schistosomiasis based on serological criteria, standard urinary tract and liver ultrasound is not informative, and it is possible to simplify clinical management of these patients [92].

MRI can be used for *O. felineus* invasion. It is shown that against the background of invasion, the MRI image has a characteristic pattern that is not typical of other diseases of the hepatobiliary tract. [93]. This method allows to determine areas with the greatest changes, which will allow to apply these methods in the future in routine diagnosis of opisthorchiasis. MRI is necessary for the diagnosis of neuroschistosomiasis in the invasion by *S. japonicum*, *S. mansoni,* and *S. haematobium* [94].

There are isolated cases in clinical practice when standard helminthological methods indicate the absence of invasion, but the endoscopic presentation allows to visualize release of liver fluke eggs into the lumen of the large intestine. One study showed that the histologic examination confirmed the diagnosis of schistosomiasis – intestinal samples contained multiple eosinophilic granulomas with *S. mansoni* eggs [95]. In individuals infested with *S. mansoni*, the effectiveness of point shear-wave elastography of the liver and spleen and correlation of its results with ultrasound parameters were shown [96].

As a result of such studies, we can talk about the auxiliary role of instrumental diagnostic methods that complement standard microscopic, serological or molecular biological methods.

OMIX TECHNOLOGIES

Thanks to the development of state-of-the-art molecular genetic methods, a significant breakthrough has been achieved in recent years in creating parasite protein libraries and studying their functions in life and adaptation to changing environmental conditions. Studies of the proteome are very important, since proteins are directly involved in implementation of genetic information and maintenance of the vital activity of the parasite, and their composition is directly determined by helminth and microorganism ecology.

The studies provided abundant data on *S. haematobium* proteins at various stages of the parasite life cycle and conducted a genomic and proteomic analysis of trematodes [97, 98]. The authors propose to use a data array to identify diagnostic markers, including the ones for assessing the intensity of invasion. As part of another study, a study of *O. viverrini* was conducted, three specific proteins were found – potential markers of *O. viverrini* [99]. A proteomic analysis of *S. mekongi* was carried out along with an analysis of their excretory / secretory protein composition [100] and proteomic screening of recombinant egg antigens to determine low-intensity *S. mansoni* invasion [30]. `

The number of such studies is growing, the array of data on proteins and helminth genes increases. As a result, personalized medicine develops, facilitating quick and individual diagnosis, identifying the degree of invasion associated with changes in the body, and developing point-of-care diagnostic methods and highly specific screening.

CONCLUSION

Improving the technologies for diagnosing trematodiasis is a significant task for public health, which is associated with spread of invasions in various regions of the world, significant financial costs for anthelmintic therapy, and monitoring of the disease in the population. Given the heterogeneity of parasitic invasions, including clinical variability, genetic diversity, the presence of different stages of pathology in the population, and the need for differential diagnosis of poly-helminth infection, there is a need to develop highly sensitive and highly specific screening

tests. The review of the literature showed that in recent years there has been pronounced research dynamics in this area. Modern microscopy technologies are being developed (including digital interpretation of data), tools for immunological analysis are being improved, classical methods of molecular genetic detection are being modified. Considerable attention of researchers is paid to the role of imaging methods in diagnosing pathological conditions associated with trematodiasis. Using omics technologies (diagnostic agents based on proteomic and metabolomic research methods) as promising diagnostic tools is discussed.

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Clinical and pathogenetic aspects of neutrophilic bronchial inflammation in asthma patients with cold-induced airway hyperresponsiveness (literature review)

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ABSTRACT

The review presents data on the effect of neutrophilic bronchial inflammation on the clinical course, external respiration, and formation of the airway response to cold air in patients with asthma. According to the results of modern studies, activation of the structural and functional state of neutrophils in a mixed inflammatory pattern is associated with an increase in disease severity, more difficult achievement of asthma control, pronounced impairment of bronchial patency due to stimulation of epithelial destruction and remodeling, and development and maintenance of cold-induced airway hyperresponsiveness.

The mechanisms activating the Th1 cytokine profile and oxidative and halogenation stress and determining the activity of neutrophils and persistence of chronic inflammation lead to oxidative damage to lung parenchyma and epithelial dysfunction, which contributes to cold-induced bronchoconstriction. Cytolysis and NETosis, acting as alternative pathways of neutrophil death in the airways of asthma patients, are considered in terms of final stages of induced activity of neutrophil lysosomes in the mixed asthma phenotype.

Keywords: asthma, mixed inflammatory pattern, neutrophils, proinflammatory cytokines, oxidative stress, cold-induced airway hyperresponsiveness

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Клинические и патогенетические аспекты нейтрофильного воспаления бронхов у больных бронхиальной астмой с холодовой гиперреактивностью дыхательных путей (обзор литературы)

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РЕЗЮМЕ

Представлены данные о влиянии нейтрофилии бронхиального воспаления на клиническое течение, функцию внешнего дыхания и формирование реакции дыхательных путей к холодному воздуху у больных брон-

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хиальной астмой (БА). Согласно результатам современных исследований, активация структурно-функционального статуса нейтрофилов при смешанном паттерне воспаления связана с утяжелением течения и более сложным достижением контроля заболевания, выраженным нарушением проходимости бронхов вследствие стимуляции эпителиальной деструкции и ремоделирования, развитием и поддержанием холодовой гиперреактивности дыхательных путей.

Механизмы активации цитокинового профиля Th1, оксидативного и галогенирующего стресса, обусловливающие активность нейтрофилов и персистенцию хронического воспаления, приводят к оксидантному повреждению паренхимы бронхов и эпителиальной дисфункции, что способствует холодовому бронхоспазму. Цитолиз и нетоз, выступающие в качестве альтернативных апоптозу путей гибели нейтрофилов в дыхательных путях астматиков, рассматриваются с позиций финальных этапов индуцированной активности лизосом нейтрофилов при смешанном фенотипе БА.

Ключевые слова: бронхиальная астма, смешанный паттерн воспаления, нейтрофилы, провоспалительные цитокины, оксидативный стресс, холодовая гиперреактивность дыхательных путей, обзор

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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BRONCHIAL NEUTROPHILS AND FEATURES OF THE CLINICAL COURSE OF THE DISEASE

Bronchial asthma (BA) has long been associated with eosinophilic inflammation. However, studies show that a number of patients have increased airway infiltration by neutrophils [1–3]. It indicates a potentially important role of neutrophils in BA development associated with phenotypic features of the disease. In a certain number of BA patients, a mixed pattern of bronchial inflammation is formed, which is characterized by a large number of eosinophils and neutrophils and accompanied by a more severe course of the disease [4, 5]. Clinically, this is manifested by persistence of symptoms, a decrease in asthma control, a decline in the lung function, and, to a greater extent, small airway patency [3].

It is estimated that an eosinophilic inflammatory phenotype is accompanied by a good response of asthma patients to treatment with inhaled corticosteroids (ICS), antileukotriene drugs (ALT), and interleukin (IL)-5 blockers [6]. Severe patients with a mixed BA phenotype have more frequent exacerbations of the disease, are difficult to treat with ICS, ALT, and require prolonged use of systemic corticosteroids (SCS) [4, 5], often responding poorly to the proposed therapy [7]. According to the literature data, neutrophilia accompanies resistance to corticosteroids and therapeutic resistance to high doses of SCS. It is associated not only with the severe course of BA, but also with the severity of its exacerbations [8].

In the period of extremely severe exacerbations and fatal attacks, the neutrophilic inflammation that dominates in the respiratory tract can lead to death [7, 8]. Thus, when studying bronchial biopsy specimens obtained from patients with severe exacerbations of BA and endotracheal intubation for respiratory failure, it was shown that during the exacerbation, the bronchial mucosa is heavily infiltrated by eosinophils and, to a greater extent, by neutrophils [9]. At the same time, a number of researchers do not exclude the presence of neutrophilia in bronchial secretion in such patients even before exacerbation due to the previous severe course of BA during treatment with high doses of ICS [10].

However, neutrophils are responsible not only for the severity of BA and resistance to ICS therapy, but are directly related to the nature of the pathological process in the respiratory tract, initiating or accompanying various exogenous reactions, for example, when exposed to cold air [3, 11]. The aggressive effect of cold leads to the formation of cold airway hyperresponsiveness (CAHR) in a significant number of BA patients (60–80%), which is accompanied by the aggravated course of BA in winter, difficulty in selecting adequate therapy due to

activation of neutrophilia, and emergence of a mixed (eosinophilic / neutrophilic) inflammatory pattern in the airways [3].

Difficulties in treating such patients are associated primarily with low efficiency of conventional anti-inflammatory therapy regimens due to the persistently high level of activated sputum neutrophils [3]. Patients with a mixed pattern of airway inflammation, even in mild BA, despite the controller therapy with ICS, more often experience respiratory discomfort and the need to use emergency drugs than patients with an eosinophilic pattern of inflammation. They also have lower values of FEV_1 and FEF_{25-75} and more pronounced bronchial lability in response to inhaled short-acting β_2-agonist and during bronchoprovocation with cold air [3]. In patients with a mixed pattern of airway inflammation, CAHR occurs 2 times more often than in patients with an eosinophilic pattern of inflammation [3]. The problem of endogenous regulation of inflammation, chemotaxis, proliferation, differentiation, apoptosis, and functioning of granulocytes in maintaining disturbed homeostasis in BA patients with CAHR remains little studied.

EXPRESSION OF PROINFLAMMATORY CYTOKINES AND THE NEUTROPHILIC LINK OF BRONCHIAL INFLAMMATION

Under the influence of a cold trigger, granulocyte neutrophils generate reactive oxygen species (ROS) and other mediators of cellular oxidation in the bronchi of BA patients [11]. The latter are represented as signaling molecules that regulate the activity of the nuclear factor NF-kB, which is one of the main transcription factors responsible for adaptive cell responses and is associated with the intensity of inflammation in BA and expression of proinflammatory cytokines [12, 13]. An elevated level of proinflammatory cytokines activates a cascade of inflammatory reactions that determine the severity of BA [13].

The impact of cold on the respiratory system of BA patients is accompanied by an increase in the concentration of IL-10, IL-5, and IL-1b in the sputum [14], which fits into the concept of antagonistic and synergistic relationships between Th2 and Th1 cytokines in BA. A pivotal role in the Th2 response of the asthma-specific transcription factor GATA-3, which is involved in the expression of Th2 cytokines, differentiation of CD4+T cells into Th2 cells, and inhibition of Th1development and mediates such components of the clinical course of the disease as CAHR and airway remodeling, has been proved [13, 15, 16].

If previously BA was classified as predominantly Th2 cell-mediated inflammation, in recent years there has been a paradigm shift, and it becomes increasingly clear that its severity is largely associated with neutrophils, which are present in large numbers in the sputum of asthma patients with CAHR [17]. The priority role in the development of neutrophilia with a mixed pattern of chronic bronchial inflammation in BA patients is attributed to the increased expression of non-Th2 cytokines, such as IL-17 and proinflammatory interferon (IFN) γ [1, 5]. They may participate in the immune response to the effects of physical and chemical environmental factors [17, 18].

The expression of IL-17 is associated with neutrophil NETosis, which often develops in BA patients along the non-lytic pathway with the formation of enucleated cytoplasts that induce differentiation of naive CD4+ helper T lymphocytes (CD4+Th0) into a subpopulation of T helper 17 (Th17) cells – producers of IL-17 [5, 19]. The enucleated cytoplasts found in the lungs in addition to neutrophil extracellular traps (NETs) result from nuclear desegmentation, disintegration of the nuclear envelope into multiple vesicles, and eruption of decondensed chromatin through ruptured neutrophil plasma membrane with cytolemma resealing. If the discarded double-stranded DNA can interact with dendritic cells (DC) localized among the epithelial cells of the airways through TLR2 receptors, causing the formation of CD4+Th2, then DC activation by cytoplasts, on the contrary, causes differentiation of Th0 cells into antigen-specific Th17 cells [5, 11].

Cytokines and activated enzymes interacting in Th17 and Th1 inflammatory responses modify the structure of the airways in BA patients, causing progression of bronchial obstruction with airway remodeling [20]. It has been known that IL-17 directly affects airway epithelial cells, fibroblasts, and smooth muscle cells [21], controlling both neutrophilic and T2 links of the inflammatory cascade [22]. Differentiation of airway Th2 cells into double positive Th2 / Th17 cells is of interest. According to *in vivo* studies, increased expression of Th2 / Th17 cells in bronchoalveolar lavage fluid is associated with the most severe airway obstruction and hyperresponsiveness, leading to an increase in corticosteroid resistance in BA [2, 18, 23].

Overproduction of Th17-related cytokines, including IL-17A and IL-17F, is thought to be a major

driver for neutrophil recruitment and activation by induction of cytokines and chemokines CXCL8, IL-6, G-CSF, GM-CSF, IL-8, CXCL1, and CXCL5, whose expression correlates with the severity of BA and neutrophilia of bronchial inflammation [24]. Thus, the neutrophilic inflammatory phenotype of BA (≥ 40% of neutrophils in the sputum) is associated with a decline in lung function, progression of disease symptoms, and an increase in the sputum concentration of IL-1b and MIP-3 alpha / CCL20 [4].

IL-8 plays an important role in neutrophilic and systemic inflammation, which primes the respiratory burst of neutrophils [25]. Among proinflammatory cytokines and chemokines, IL-8 preactivates respiratory reactions in cells [26]. As a chemokine, IL-8 plays a key role in neutrophil chemoattraction: it stimulates the migration of neutrophils from the bloodstream to the site of inflammation, increases the concentration of intracellular Ca^{2+} in them, which ensures the movement of leukocytes and activates the pentose phosphate pathway in these cells, causing the production of free radicals and degranulation and exocytosis of neutrophilic enzymes [27]. Integration of IL-8 with IL-1, GM-CSF, TNFα, and other proinflammatory cytokines constitutes a cytokine field that activates neutrophils [27], which, in turn, independently synthesize and produce neutrophilokines (GM-CSF, TNFα, IL-1, IL-6, IL-8), involved in the cooperation of phagocyte system cells and acting in a paracrine manner on macrophages and in an autocrine manner on neutrophils [28].

There is an opinion that neutrophils stimulated by IL-8 in BA lead to airway eosinophilia by modifying the migration of eosinophils through the basement membrane of the bronchial mucosa. Thus, due to increased migration of eosinophils induced by the neutrophil + IL-8 complex, the possibility of disease exacerbation is multiplied [29].

Expression of IL-8, TNFα, and other chemokines is controlled by NF-kB, which has a stimulatory effect on numerous genes involved in immune, acute phase, and inflammatory responses and airway smooth muscle responses [12]. In turn, NF-kB is activated by a direct synergistic effect of TNFα and the bifunctional enzyme CD38, which is expressed in cells of the immune system, as well as in vascular and bronchial leiomyocytes. The CD38 molecule, which combines the activity of adenosine diphosphate (ADP) ribosyl cyclase and cyclic ADP-ribose hydrolase (cADPRH), serves as a marker of immunopathological processes characteristic of BA [30]. TNFα-induced expression of CD38 potentiates the expression of multiple proinflammatory genes, increasing smooth muscle contractility, which leads to increased airway resistance to air flow and contributes to the development of bronchial obstruction [30, 31]. Under the influence of cold air in patients with CAHR, there is an increase in the concentration of IL-1b, IL-8, and TNFα [17]. There is a close relationship between the concentration of TNFα in the sputum and the severity of bronchoconstriction in response to inhalation of cold air [17].

In addition, it is likely that in patients with CAHR, there is a decrease in antiviral immunity due to the suppression of the antiviral and immunomodulatory activity of IFN through the activation of NF-kB and the expression of genes encoding proinflammatory cytokines [32]. This may cause persistence of infection in the airways and is considered as one of the potential independent mechanisms in the uncontrolled course and exacerbation of the disease [2, 13].

RESPIRATORY BURST AND AIRWAY MYELOPEROXIDASE ACTIVITY

The proinflammatory cytokines GM-CSF, TNFα, and IL-8 modulate the activity of NADPH oxidase in neutrophils via priming. NADPH oxidase is a multicomponent enzyme system catalyzing NADPH-dependent reduction of oxygen to the superoxide anion O_2^{2-}, i.e., capable of oxidizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) and redeploying electrons from NADPH to molecular oxygen [26]. This process (respiratory burst) is regulated by many receptor and non-receptor reactions, which culminate in conformational changes in NADPH oxidase components and their readiness to interact with one other [26, 33].

The assembly of a single NADPH oxidase complex occurs at the center of the respiratory burst and is triggered by the activation of cell receptors, kinases, and guanosine triphosphatases, leading to phosphorylation and membrane relocation of oxidase components [26, 33]. Taking the first electron, an oxygen molecule turns into a superoxide radical anion $O_2^{\bullet-}$; with further reduction, either an H^+ ion is added to form a hydroperoxide radical $HO_2^{\bullet}$, or an electron is formed to shape a superoxide anion $O_2^{2-\bullet}$.

During the respiratory burst, the aggressive superoxide anion radical is removed spontaneously or under the effect of superoxide dismutase (SOD). Either an electron or an H^+ ion is attached to $O_2^{\bullet-}$ and a hydroperoxide anion HO_2^- is formed, which

is then reduced to a more stable hydrogen peroxide H_2O_2. At this stage, myeloperoxidase (MPO) released from neutrophil lysosomes is incorporated into the respiratory burst, converting oxidative stress into halogenation stress based on synthesis of highly reactive halogen-containing compounds [34, 35]. Hypochlorous acid (HOCl) is the main product of the interaction between MPO and H_2O_2 – the most active precursor of free radicals among reactive halogen species (RHS) [34, 35].

The cascade of free radical reactions caused by oxidative stress causes disruption of the structure and function of biomembranes and damage to all vital molecules in cells of the respiratory tract, leading to disorganization of the lung parenchyma and stroma [36]. MPO involved in the respiratory burst of neutrophils, when interacting with H_2O_2, catalyzes the oxidation of halide ions (Cl^-, Br^-, I^-), contributing to the production of hypohalogenites, hypohalogenite derivatives (HOCl, HOBr, HOI), and their ionized forms (hypochlorite, hypobromite and hypoiodite) [36]. As a result of the occurring reactions, a link between oxidative stress and halogenation stress is provided [34, 37].

MPO from azurophilic granules of neutrophils is secreted into the intercellular space as a result of degranulation during the respiratory burst. In asthma patients with CAHR, neutrophil degranulation in sputum occurs to the level of destruction [11]. Total degranulation, which, as a precursor of destruction, realizes the maximum oxidative capacity of neutrophils at the peak of the respiratory burst, is preceded by the activation of oxidases, which is proportional to the needs of bronchial inflammation in ROS and RHS. Enhanced synthesis and intragranular deposition of MPO, followed by extracellular exocytosis of the enzyme and products of its catalytic activity, prolong oxidative stress and provoke inflammatory damage to the epithelial parenchyma, accompanying the bronchial response to cold exposure.

Accumulation of peroxidase in neutrophils, stimulated by the accelerated utilization of HOCl, hypochlorite anion, and other ionized forms of hypohalogenites in the bronchial matrix, ends with functional depletion of cells, depletion of peroxidase-positive granules, disruption, and cytolysis with primary destruction of the cytoplasm, then – of the nucleus and cell lysosomes [11]. A differential sign of transformation of physiological degranulation (as a link in cellular adaptation to cold stress) into pathological destruction is vacuolization and fragmentation of the neutrophil cytoplasm in BA [38].

In contrast to degranulation, in which structure-preserving granules with enzymes deposited in them are secreted into the intercellular space, during neutrophil destruction, labilization of lysosome membranes occurs, and the lysosomal matrix penetrates into the environment. In sputum smears, the concentration of MPO in granulocytes is determined by the oxidation reaction of benzidine in the presence of H_2O_2 [38]. This reaction shows dense, diffuse, or granular location of a black-colored product in the cytoplasm of granulocytes, which marks peroxidase activity under conditions of intense synthesis and accumulation of the enzyme in granules. Due to the compact arrangement of granules larger than 0.3–0.4 μm, the cytoplasm of neutrophils is either filled with an intensely stained diffuse and granular material that masks or incompletely masks nuclear segments, or acquires a homogeneous black color, with which benzidine, oxidized by MPO, prevents detection of the cell nucleus [38].

Against the background of the clear and vacuolated cytoplasm in neutrophils during degranulation and destruction, peroxidase-positive inclusions marked with benzidine are present in the form of sparse or single, softly colored small granules, up to 0.2–0.3 μm in length [38]. The low concentration of MPO in neutrophils is regarded as a consequence of increased utilization of the enzyme during the generation of RHS, degranulation, destruction, and cytolysis of cells associated with respiratory burst and production of free radicals. All of the above stimulates the persistence of inflammation and the formation of an excessive airway response to cold exposure and leads to a decline in the lung function and a decrease in asthma control [3, 38].

NEUTROPHIL PROFILE AND DESTRUCTION OF BRONCHIAL EPITHELIUM

The most pronounced destruction of the epithelium is observed in patients with BA with a mixed inflammatory pattern due to ongoing intense destructive processes in the neutrophil pool [2, 39]. Destruction of the bronchial epithelium has a negative impact on airway patency and control of the disease, contributing to the formation of an increased response of the bronchi to cold exposure with the emergence of CAHR [39].

The generation of ROS and RHS exported by neutrophils is considered as a cause of free radical damage to mitochondrial cristae and destruction of the endoplasmic reticulum of the bronchial epithelium,

followed by cell death [40]. It is assumed that in this case, apoptotic signals are transmitted to the epithelium not via a direct pathway, from ligation of the death receptor to activation of the caspase cascade and cell death, but via a pathway mediated by de-energization of epitheliocytes [40]. Actively developing epithelial dysfunction in BA is associated with low expression of antioxidant defense factors, in particular, SOD, which contributes to an increase in the susceptibility of epithelial cells to the aggressive effect of oxidants [41]. Activation of the neutrophilic link of bronchial inflammation [42] is also associated with more pronounced mucociliary dysfunction in patients with BA who respond to cold air exposure [43]. Thus, in patients with CAHR, a close relationship was found between disorders in the structural organization of goblet cells, a large number of sputum neutrophils, and the severity of cold-induced bronchospasm, which served as a risk factor for escalation of mucociliary dysfunction [43].

According to a number of sources, oxidative damage to the airway epithelium leads to exocytosis from intraepithelial nerve endings of C-fibers (non-adrenergic / non-cholinergic) in substance P and neurokinins A and B – neurotransmitters with pronounced bronchoconstriction and vasodilator effects [41]. The produced substances activate mast cells, macrophages, and T and B lymphocytes and serve as chemoattractants for eosinophils and neutrophils. Inhibition of the vasoactive intestinal peptide (VIP), synthesized by neurosecretory cells of the bronchial epithelium, occurs, production of PgE_2 is impaired, surface cell receptors, proinflammatory cytokines, chemoattractants, and GM-CSF are expressed [41].

There is evidence that epithelial cells from bronchial biopsies of patients with BA are characterized by a significant increase in the production of GM-CSF and expression of proinflammatory IL-6 and IL-8, the concentration of which increases drastically under the influence of exogenous factors [2]. Airway exposure to cold air in patients with CAHR is accompanied by overproduction of IL-8 and TNFα, an increase in cytosis, and a rise in the proportion of neutrophils in the sputum, leading to a cytokine imbalance associated with bronchospasm [17]. In parallel, there is a decrease in the number of cells of the bronchial epithelium, which is associated with induction of Th1 cytokines and a shift in humoral inflammation to the Th1 cytokine profile. In addition, the decrease is associated with epithelial destruction, parenchymal modification of the airways, expression of the NF-kB factor, and release of proinflammatory cytokines during oxidative damage [2, 17]. The bidirectional behavior of NF-kB, which induces cytokines and is induced by cytokines, controlling the expression of genes encoding proinflammatory cytokines, is a fundamental point that brings together oxidative functions of neutrophils and proinflammatory activity of the airway epithelium, which makes an important contribution to the pathogenesis of airway hyperresponsiveness and remodeling [30].

In addition to oxidative damage, neutrophils produce proteases. The combined effect of both factors causes direct non-specific damage to lung tissues. The production of matrix metalloproteinases (MMPs) contributes to the degradation of the extracellular matrix in BA [2]. As it turned out, MMP-9 can degrade components of the extracellular matrix, including type IV basement membrane collagen, which makes a significant contribution to the integrity of the endothelium and (or) epithelium [2]. Studies show that remodeling is caused by the differentiation of mesenchymal cells of the bronchial subepithelial layer into myofibroblasts [44] with thickening of the reticular basement membrane, interstitial fibrosis, and atrophy of the surface epithelium [45]. Therefore, activation of the neutrophilic component of granulocytic inflammation in patients with BA is not only associated with the destruction of the airway epithelium and airway hyperresponsiveness, but also manifests escalation of airway remodeling, transferring BA into a more severe form.

CYTOLYSIS AND NETOSIS AS VARIANTS OF DEATH OF BRONCHIAL NEUTROPHILS ALTERNATIVE TO APOPTOSIS IN BA

If destruction of granulocytes is a process that reflects the general biological patterns in the dynamics of their functional activity [46], then intensification of destruction and cytolysis are interpreted as the result of stimulated functional activity associated with progressive inflammatory alteration, lysis of the cell membrane, cell isolation, and necrosis [47].

In BA, granulocytes show an IL-5-dependent increase in the expression of *bcl-2* genes, which causes suppression of apoptosis, prolongation of the damaging activity of granulocytes, and an increase in the proportion of dying cells [48]. Antiapoptotic factors regulating apoptosis are formed in the focus of inflammation, while the intermembrane space of neutrophil mitochondria contains proapoptotic

proteins that penetrate into the cytosol during apoptosis [49].

In respiratory diseases, mitochondrial membranes are the most vulnerable link in the pathogenesis of damage to subcellular structures. Changes in mitochondria cause disturbances in biological oxidation and cellular respiration, lead to a decrease in the intensity of energy exchange, ATP deficiency, and destruction of other organelles and the cell as a whole [5]. A decrease in the ability of neutrophils to undergo apoptosis correlates with destruction of mitochondrial cristae and indicates de-energization of cells [46, 49], while in patients with BA, there is a twofold (by 2.1 times compared with controls) increase in the pool of low-energy cells characterized by destruction of cristae and outer membrane mitochondria [46]. Activation of lysosomal acid phosphatases is also directly involved in the stimulation of neutrophil cytolysis, which causes an increase in the activity of neutrophil acid phosphatase by 22.0% in BA [49].

It is most likely that the mechanism responsible for the decrease in the number of neutrophils in the airways of asthma patients, in addition to cytolysis, includes such an alternative to apoptosis as "classical" NETosis, a process of programmed oxygen-dependent cell death aimed at producing highly active neutrophil extracellular traps (NETs) (an important tool for phagocytosis and elimination of pathogens and inflammation products) in response to the effects of stimuli [50–53]. Proinflammatory agents stimulating NETosis can include H_2O_2, bacterial lipopolysaccharides, phorbol myristate acetate, IL-8, and the cleavage product of the fifth complement component during its activation in the blood serum (C5a), but only after priming of neutrophils with interferons (IFNγ + C5a, IFNα+C5a) or GM-CSF (GM-CSF+C5a) [51]. It is believed that almost all neutrophils are capable of NETosis due to the presence in these cells of regulatory mechanisms for starting a program that ends with the formation of NETs [50].

The formation of NETs begins with neutrophil priming, triggering of the NADFH oxidase complex, respiratory burst, and generation of ROS that induce neutrophil elastase and PAD-4, converting arginine and methylarginine residues into citrulline in histones of the nucleus [51, 54]. As a result, chromatin decondensation occurs with a simultaneous violation of the structural integrity of the membranes in cytoplasmic granules [51, 53]. When decondensed chromatin (DNA strands, histones) is mixed with lysosomal granule enzymes, reticulated NETs are secreted into the extracellular space [50–53].

NETs include MPO, neutrophil elastase, cathepsin G, gelatinase, antibacterial peptides, histones, humoral pattern recognition receptor pentraxin 3, and peptidoglycan recognition proteins [51]. During the formation of NETs by mixing nuclear chromatin with the contents of lysosomal granules, the activated neutrophil still retains its viability and functional activity. Death of an activated ("damaged") cell occurs only after the release of reticulated structures into the extracellular space [49]. Therefore, we are talking about the existence of "vital" NETs that play a role in non-type 2 inflammation, correlate with the level of IL-17 and bronchoalveolar lavage neutrophilia [5, 19], and are found in the airways of patients following attacks of rhinovirus infections that provoke BA exacerbations [19]. Vital release of chromatin by neutrophils primed with proinflammatory cytokines GM-CSF and IL-5/IFN-γ and retaining their effector functions for some time after the formation of NETs develops much faster than the classical ("suicidal") NETosis. However, in any case, after the destruction of the pathogen and the completion of the infectious process, NETs should be eliminated via DNase I and lysis by macrophages. Overproduction of NETs, as well as disturbances of mechanisms for their elimination, for example, in the absence of DNase I, can cause the development of inflammation or autoimmune pathology [55].

Thus, even alternative pathways of neutrophil death in the airways – cytolysis as an outcome of enzymatic activity of cells induced by the respiratory burst and NETosis as a tool for the secretion of inflammatory mediators by ROS-stimulated NETs – can be considered from the standpoint of their role in the pathophysiological mechanisms of neutrophil activity in a mixed inflammatory BA phenotype.

Based on the above data, some potential areas for further research on the mixed inflammatory pattern in asthma patients with CAHR can be identified. It is advisable to study NETs as a marker of IL-17-mediated neutrophilic inflammation, which negatively affects clinical manifestations of BA, since the inducing effect of NETs on the differentiation of CD4+ T cells into Th17 cells and production of IL-17 was established [19]. As it is known, IL-17A and TNFα induce the production of the neutrophil chemokine CXCL-8 by the bronchial epithelium due to impaired inhibition of CXCL-8 expression by an internal repressor protein, which causes hyperreactivity and dysfunction of the

epithelium [56]. Normalization of the cytoplasmic translocation of the CXCL-8 repressor protein in the bronchial epithelium is a potential therapeutic target in neutrophilic BA. In addition to determining the level of IL-17 (IL-17A, IL-17F) in the sputum and bronchial epithelium of asthma patients with CAHR, which plays a key role in neutrophilic inflammatory phenotype, it is necessary to identify the components of the IL-17 signaling pathway that activates the transcription of IL-17A target genes encoding proinflammatory cytokines. The key component of the canonical IL-17 signaling pathway is the TRAF6 regulator (factor 6 associated with the tumor necrosis factor receptor) whose modification leads to the activation of the NF-kB and MAPK pathways.

Finally, taking into consideration that recruitment of neutrophils to the airways is associated with an increase in the level of proteolytic enzymes, including neutrophil elastase and MMP-9 [57], it is promising to study the activity of these enzymes in the sputum, since they cause destruction of collagen fibers and are involved in airway remodeling. High levels of neutrophil elastase and MMP-9 can be considered as markers of proteolysis activation, which intensifies airway remodeling and is controlled by the effects of IL-17A.

As potential targets in BA therapy in patients with CAHR with a mixed inflammatory pattern, development of biological preparations based on monoclonal antibodies should be considered. These include inhibitors and antagonists of Th17 cells, IL-17, TNFα, IL-8, IL-6, NF-kB-dependent proinflammatory cytokines, and Th1 immune responses.

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Global best practices in recruiting and retaining healthcare workers in rural areas (literature review)

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ABSTRACT

A significant issue for global healthcare is recruitment and retention of doctors and nurses, especially in rural areas. It threatens continuity and accessibility of medical care for a large segment of the population.

The aim of this article was to summarize currently available data on healthcare recruitment practices, particularly in rural areas, and key factors influencing retention of healthcare professionals. This will allow to develop evidence-based strategies for recruitment and retention of healthcare workers in the Russian Federation and reduce personnel shortage. International and Russian full-text articles were searched for in PubMed, ScienceDirect, Cochrane Library, Google Scholar, and eLibrary databases.

All the studied factors influencing recruitment and retention of healthcare professionals in rural areas were grouped into four main categories: financial, social, professional, and personal. Modern healthcare recruitment strategies were divided into three groups: financial, organizational, and instructional.

The review results suggest that the Russian Federation uses the majority of global strategies to recruit and retain healthcare professionals in rural areas. However, there are some activities that have not been adopted in our country. They may be included in healthcare management practices to increase the effectiveness of regional programs for development of human capital in healthcare.

Keywords: recruitment, retention, strategies, healthcare workers, rural areas, overview

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Мировые практики привлечения и удержания медицинских работников в сельских районах (обзор литературы)

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РЕЗЮМЕ

Привлечение и удержание врачей и среднего медицинского персонала, особенно для работы в сельских районах, является глобальной проблемой системы здравоохранения, ставящей под угрозу непрерывность и доступность оказания медицинской помощи значительной части населения.

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Целью данного исследования является обобщение имеющихся на сегодняшний день данных о практиках привлечения медицинского персонала, прежде всего в сельскую местность, и ключевых факторах, влияющих на удержание работников в сфере здравоохранения, что в будущем поможет разработать научно обоснованные мероприятия и подходы к привлечению и удержанию медицинского персонала и тем самым позволит сократить кадровый дефицит в системе здравоохранения Российской Федерации. Поиск отечественной и зарубежной литературы проведен в базах данных PubMed, Science Direct, Cochrane Library, Google Scholar, eLibrary.

Все изучаемые в настоящем исследовании факторы, влияющие на привлечение и удержание медицинских работников в сельской местности, объединены в четыре основные группы: финансовые, социальные, карьерные (профессиональные), личностные. Современные стратегии по привлечению персонала можно разделить на три направления: финансовые, организационные и образовательные.

Результаты обзора свидетельствуют, что в Российской Федерации реализуется большая часть мировых практик по привлечению и удержанию специалистов в сельских районах. Однако существуют мероприятия, которые не нашли своего применения в нашей стране. Данные практики могут стать дополнительными при формировании региональных программ по развитию кадрового потенциала здравоохранения, увеличивающие их эффективность.

Ключевые слова: привлечение, удержание, медицинские работники, сельские районы, стратегии, обзор

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов в связи с публикацией данной статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Continuity and accessibility of medical care worldwide are threatened by a shortage of healthcare professionals in rural areas [1]. The World Health Organization (WHO) estimates a projected shortfall of 17 million health workers by 2030 [2]. The World Economic Forum estimates that by 2030, there will be a shortfall of 14.5 million healthcare professionals [3]. According to Rosstat data, there was a shortage of 24.7 thousand doctors and 127.1 thousand paramedical staff in the Russian Federation in 2021 [4]. Most researchers agree that causes of this may include problems in medical education, low social status of physicians, high workload, poor working conditions, and low salary [5]. Besides the absolute shortage of healthcare workers, uneven distribution of health professionals by specializations and geographical areas is registered [6, 7].

To solve this problem, strategies on recruiting and retaining health workforce are being developed and implemented around the world [8, 9]. Since motivation of healthcare professionals is influenced by a complex of interrelated factors, a well-chosen combination of various measures is required for their effective recruitment and retention [10].

The aim of this article was to summarize currently available data on healthcare recruitment practices, particularly in rural areas, and study the key factors influencing retention of healthcare professionals. This will allow to develop evidence-based strategies for recruitment and retention of healthcare workers in the Russian Federation and reduce personnel shortage in the future.

The PubMed, ScienceDirect, Cochrane Library, Google Scholar, and eLibrary databases were used to search for publications. The following keywords were used: “attract”, “recruitment”, “retention”, “health workers” etc. We considered articles published in 2015–2022. Several issues required a more detailed data analysis. A total of 3,890 sources were analyzed, from which 540 studies with representative samples were chosen. The final version of the review included 65 articles on strategies to recruit and retain medical workers.

FEATURES OF RECRUITMENT AND RETENTION OF HEALTHCARE PROFESSIONALS IN RURAL AREAS

The decision of specialists to stay in or leave rural areas is complex and influenced by a variety of factors [11]. Based on the examination of published studies, all factors can be divided into four categories.

Financial factors include salary, training reimbursement, benefits, and allowances. In most studies, salary was one of the key incentives for specialists to work in rural medical facilities. According to J. Witt et al. (2017, *n* = 2, 478, Canada), salary, working hours, and frequency of home visits were the most important factors in considering a place of work [12]. N. Nurelhuda et al. (2018, *n* = 455) showed that medical students thought that regional healthcare should provide them with a scholarship throughout their studies [13]. In a similar study in Indonesia (2016, *n* = 400), F. Efendi et al. (2016) obtained comparable data and revealed the importance of modern, high-quality equipment [14]. Based on the findings of S.C. Okoroafor et al. (2021, *n* = 198, Nigeria), higher salary increased the probability of working in rural areas by 2.7 times, while accommodation or housing allowances increased the odds by 4 times [15].

Following the outcomes of an in-depth interview by L. Berman et al. (2021), Malaysian doctors (*n* = 472) would be 6.67 times more likely to prefer a rural career if incomes were raised by 50% (95% confidence interval (CI): 5.66–8.08). [16]. V.A. Smiianov et al. (2017, *n* = 167) found that most doctors would agree to an increase in workload for an additional fee, but they were unlikely to change their place of work for a higher-paying job [17]. O.A. Doshchannikova (2020, *n* = 561) discovered that 16.8 ± 1.6% of doctors decided to leave the rural are after the end of the Socioeconomic Support Program. "Low salary" (39.3 ± 2.1%) and "family circumstances" (21.3 ± 1.73%) were the most common explanations [18]. A survey of 259 doctors and 215 medical students in Zambia by M.L. Prust et al. (2021) found that participants would prefer to work in hard-to-reach areas if they were offered accommodation in rural areas (odds ratio (OR) = 5.04, 95% CI: 4.12–6.18), accommodation in the city (OR = 2.21, 95% CI: 1.86–2.62), or an allowance after four years of work in a rural medical facility (95% CI: 1.69–2.33) [19].

Social factors include living and working conditions. According to the survey of nurses carried out in Australia by M. Prengaman et al. (2017), availability of materials and equipment for work had the greatest effect on recruitment and retention of nurses in rural areas [20]. S.C. Okoroafor et al. (2022, Nigeria, *n* = 145) revealed that availability of school education for children increased the chances of working in rural areas by 6.17 times, provision of accommodation – by 14.6 times, and improvement of living and working conditions – by 14.4 times [21]. The key factors in the study by U. Lawan et al. (2017, *n* = 262, Nigeria) were infrastructure and equipment (92.3%), improvement of living conditions (91.2%), availability of clean water and electricity (91.5%), and schools for children (91.5%) [22]. According to S.B. Boadi-Kusi et al. (2018, *n* = 333, Nigeria), improved living conditions were major factors for 71.2% of respondents in their decision to move to rural areas [23].

The study by L. Berman et al. (2021) showed that accommodation, the quality of the working environment, and workload were the most important factors in choosing a place of work. Respondents also were 2.04 times more likely to select a job in rural areas with better living conditions (95% CI: 1.71–2.44) and 1.7 times more likely to choose a job with a better workspace environment (95% CI: 1.47–1.96) [16].

Professional factors include continuing education opportunities, scientific and educational activities, professional development at the expense of the employer, career growth, and professional community support [24–26]. V.A.T. Nguyen et al. (2020, 2021, *n* = 167) [25, 26] demonstrated the significance of these factors in Vietnam**.** In a survey by S. Mollahalilolu et al. (201, *n* = 1,340), rural health professionals said that remote location of a medical facility hindered development of their professional skills [27]. Career growth was essential to 71.3% of participants in the research by S.B. Boadi-Kusi et al. (2018) [23]. According to O.A. Doshannikova (2020), 19.7 ± 1.68% of doctors would leave the rural area due to "a lack of career growth and professional advancement opportunities" [18]. G.Yu. Okuneva et al. (2021) found that 35.9% of senior medical students (*n* = 280) did not want to work in rural regions due to a lack of professional development and career growth [28].

However, C. Morken et al. (2018, *n* = 16) showed that professional advancement opportunities had no significant influence on retention of medical workers in rural areas [29]. According to S.C. Okoroafor et al. (2021,) Doctors and Candidates of Sciences were 4% less likely to work in remote regions [15].

Personal factors include demographic information (age, gender), education level, marital status, place of birth, and place of residence.

According to the findings of an Israeli study (n = 6,673), rural healthcare workers were 17 times more likely to stay in the rural area for work (95% CI: 6.8–43.8), while those without work experience in remote areas were 28% less likely to continue working in rural areas (95% CI: 0.66–0.92). Furthermore, women were significantly less likely to choose jobs in rural areas than men (OR = 0.66, 95% CI: 0.56–0.78) [30].

According to the survey of 184 students from West Africa by C.S. Sidibé et al. (2019), a place of residence (p = 0.003) and location of an educational organization in rural areas (p = 0.03) are associated with a stronger inclination to stay and work there [31]. L. Berman et al. (2021) discovered that students who had previously lived in rural regions (OR = 3.4, 95% CI: 1.71–6.79) and those who had an employer-sponsored training agreement were significantly more likely to say that they would continue working there (OR = 3.66, 95%CI: 2.22–6.04) [16]. M.L. Prust et al. (2021) confirmed the importance of having work experience in rural regions when choosing a place of work [19].

Marital status is another significant factor in making a decision to work in rural areas [32–34]. When personal files of 30,569 Iranian family medicine doctors were examined, it was found that the marital status was a significant predictor of choosing a job in the rural area (OR = 1.34, 95% CI: 1.25–1.43) [33]. Furthermore, S.C. Okoroafor et al. (2021) discovered that practitioners with children were 6 times more likely to work in rural medical facilities [15].

Table

Factors influencing recruitment and retention of healthcare professionals

Author	Year	n*	Country	Factors influencing recruitment and retention of healthcare workers
Witt et al.	2017	2,487	Canada	Salary, working hours, and the frequency of doctor's home visits
Nurelhuda et al.	2018	455	Sudan	Providing scholarships during the study period, improving the equipment of medical organization
Okoroafor et al.	2021	198	USA	Accommodation, bonuses, improved working conditions
Berman et al.	2021	472	South Africa	Accommodation, workload, working conditions, career growth, living in a rural area
Efendi et al.	2016	400	Indonesia	Education scholarships, salary, working conditions, modern equipment
Ashkenazi et al.	2019	736	Israel	Working conditions, professional advancement, partner's employment, parents' living in the rural area
Nguyen et al.	2020	167	Vietnam	Continuing medical education, advanced training, parents' living in the rural area
Prengaman et al.	2017	16	Australia	Support from the professional community, availability of necessary resources and equipment
Morken et al.	2018	16	USA	Job satisfaction, work – life balance, support from family and professional community
Taati Keley et al.	2016	6,673	Iran	Male gender, living in the village, practical training and work experience
Boonluksiri et al.	2018	10,018	Thailand	Rural location of an educational institution
Ehsani-Chimeh et al.	2018	30,569	Iran	Salary, marital status, developed infrastructure
Flores et al.	2021	102	Philippines	Working conditions, marital status, professional advancement, salary, accommodation
Prust et al.	2019	474	Zambia	Work experience in rural areas, improvement of living conditions / provision of accommodation, scholarships
Sidibé et al.	2019	186	West Africa	Living in a village, location of an higher education institution in the rural area
Mollahaliloðlu et al.	2015	1,340	Turkey	Career advancement and professional development opportunities
Okoroafor et al.	2022	145	Nigeria	Working and living conditions, availability of high-quality school education in rural areas
Lawan et al.	2017	261	Nigeria	Developed infrastructure, available drinking water and electricity, schools for children
Boadi-Kusi et al.	2018	337	Gana	Financial incentives, improvement of living conditions, career growth
Doshchennikova O.A. et al.	2020	561	Russia	Salary, career and professional growth, family ties, living conditions
Okuneva G.Yu. et al.	2021	280	Russia	Financial incentives, accommodation, rural lifestyle, opportunities for professional and career development, improved working conditions

* number of participants

Best practices in recruiting and retaining healthcare professionals in rural areas

Based on the analysis of publications, all strategies may be grouped into three broad categories: financial, organizational, and educational.

Financial strategies to recruit and retain healthcare professionals

There are three ways to use financial measures to recruit and retain healthcare workers. The first way is a long-term monthly bonus in the form of a fixed

salary increment. In India, for example, such a system was implemented: by 2011, 1,319 specialists had been employed, and the number of vacant positions in medical organizations decreased from 90 to 45% [35]. In Bangladesh, financial benefits in the amount of 33% of the base salary rate, but no more than $ 38 per month, are given to physicians working in rural areas [36]. Tanzania pays daily allowances and subsidizes housing, and measures are taken to accommodate physicians arriving in sparsely populated areas in houses of other medical practitioners before the former rent accommodation [37].

The second type of financial measures is a scholarship for students who want to pursue specialized training. According to Z. Marsh et al. (2019), more than 220 nurses were recruited in the United Kingdom within three years after the program implementation [38]. There is also a one-time payment option available, with a minor pay rise. In China, a program was implemented that takes into consideration work experience in rural areas: with work experience of more than 5 years, a bonus of 50–200 thousand yuan is paid; graduates of medical schools who have worked in rural areas for more than 1 year receive a bonus of 10–30 thousand yuan. Since the program implementation, the proportion of healthcare professionals who are willing to work in rural areas has increased from 1.09% in 2013 to 6.49% in 2016 [39].

Organizational strategies for recruiting and retaining healthcare workers

According to the findings of the study, financial benefits were secondary and less effective than non-financial factors [29]. According to C. Marchand et al. (2017), strategies focused on professional recognition and job satisfaction are more significant than financial ones [40]. L. Tudor Car et al. (2021) obtained similar results in their research [41]. According to N. Sirili et al. (2018), developing individual plans for professional and career growth is effective in retaining practitioners in rural medical facilities [37]. Mentorship programs also have a significant influence on retention of healthcare professionals in remote areas. According to D.M. Gumede et al. (2021), implementation of mentorship programs in 2002 enabled to recruit more than 300 doctors to work in hard-to-reach areas of the Republic of South Africa [42].

According to R. Schaefer et al. (2021), implementation of a flexible work schedule and an opportunity of part-time employment for doctors and paramedical staff allowed to retain the most efficient workers, prevent professional burnout, and guarantee stability and continuity of work [43]. A similar strategy was successfully implemented in Australia: according to the findings of the research by V.P. Weale et al. (2017, $n = 108$), it allowed to improve job satisfaction and maintain a work – life balance [44]. Professional development opportunities in medical organizations had a substantial effect on job satisfaction in the study conducted by A. Honda et al. (2015, Mozambique) [45].

Educational strategies for recruiting and retaining healthcare professionals

It is essential to meet the need of healthcare workers for training and education throughout their careers. In the project described by R. Shah et al. (2021), 611 healthcare workers were distributed to five hard-to-reach areas of Guinea in 2017. Of them, 18% of workers were going to leave after 12 months; with the remaining employees, semi-structured interviews were conducted, showing that continuing education opportunities motivated doctors to work in hard-to-reach areas [46].

The Republic of South Africa implemented an educational program for physicians at rural medical organizations (decentralized), which is focused on physicians' needs. By 2020, nine participants had graduated and were still working in hard-to-reach areas [47]. Since 2007, a training course for rural students at rural medical colleges has been established in Wisconsin, USA: 51% of students continue to work in rural areas [48]. Such educational programs have been used effectively in Canada, Australia, the Philippines, the Republic of South Africa, Mali, Thailand, and other countries [49–51].

T. Woolley et al. (2018, $n = 283$) revealed that 31% of graduates of socially responsible medical education institutions based at rural medical facilities continue working in remote areas, compared to 7% of graduates of ordinary higher education institutions ($p < 0.001$) [52]. A study conducted by M.R. McGrail et al. (2016, $n = 610$) in Austria discovered a significant correlation between respondents' birth and education in a rural area and their choice of a successive place of work: 74–91% of respondents with a rural place of birth and 87–95% of those educated in rural areas managed to remain in that area for the first five years after accreditation, whereas this effect was amplified when combined with a rural place of birth (OR 24; 95% CI: 13–43) [53]. In the study by P. Boonluksiri

et al. (2018) that included 10, 018 physicians, 21% of whom lived in the rural area, 5, 774 persons (57.6%) decided to work in rural medical organizations; those who completed a similar program in Thailand were considerably more likely to stay in rural regions compared to other peers (62.3% and 49.0%, $p < 0.001$) [54].

Since 2014, a model of early application and priority enrollment in residency based at rural medical facilities has been implemented in Norway. According to M. Gaski et al. (2018, $n = 388$), the proportion of graduates who applied for a residency program ahead of time and decided to work in remote areas (29%) was twice as big as the percentage of other respondents (15%) [55].

BEST PRACTICES IN RECRUITING AND RETAINING HEALTHCARE WORKERS IN RURAL AREAS IN THE RUSSIAN FEDERATION

Most of the described strategies are successfully implemented in the Russian Federation. The program Zemsky Doctor (Rural Doctor) has been implemented since 2012, while Zemsky Feldsher (Rural Paramedic) has been implemented since 2015. Medical workers (doctors, paramedics) who arrive in a rural area, an urban-type settlement, or a small town with the population of up to 50 thousand people receive a one-time payment of up to two million rubles[1]. According to V.M. Chernysheva et al. (2022), the implementation of the Zemsky Doctor and Zemsky Feldsher programs in the Siberian Federal District did not lead to an increase in staffing. From 2016 to 2020, availability of physicians decreased from 15.1 to 13.5 (per 10,000 population), and availability of paramedical personnel reduced from 59.1 to 54.2 (per 10,000 population). The comparison of participation in the program with staffing was the study limitation; the number of workers who left the profession was not taken into consideration [56].

According to D.A. Bugaev et al. (2019), the largest number of agreements within the Zemsky Doctor program were signed in the Stavropol Krai in 2012 ($n = 259$), and the smallest – in 2016 ($n = 70$) [57]. According to the study by V.V. Zubkov et al. (2019), during the program implementation (2012–2017), the shortage of physicians in the rural areas of the Khabarovsk Krai decreased by 30%, and 202 doctors were recruited in medical facilities in all districts of the region [58]. According to M.V. Kinchagulova (2018), the total number of physicians in rural areas of the Tyumen Oblast increased by 30.2% (844 persons) in 201–2021 as a result of the program implementation [59]. According to the findings of A.V. Danilova (2018), 466 medical practitioners moved to rural areas of the Voronezh Oblast from 2012 to 2017, 74 people moved to work settlements, and 23 – to urban-type settlements [60]. From 2012 to 2021, 52,000 healthcare workers were involved in the program implementation. Approximately 6,000 workers were recruited in 2021 due to one-time payments.

Other types of support are also being implemented in a number of Russian regions. Since 2013, the Leningrad Oblast has been offering financial benefits in the form of annual payments totaling 120 thousand rubles. In 2017, 530 people received such benefits among hospital specialists, such as anesthesiologists, resuscitators, neonatologists, psychiatrists, psychotherapists, and phthisiologists [61]. Physicians in the Kaliningrad Oblast receive a one-time payment in the amount of 300 to 900 thousand rubles[1] after their first employment in a government-funded medical organization. Specialists who live in rural areas are reimbursed for accommodation and utility bills and are offered mortgage assistance programs and partial rent coverage in Leningrad, Pskov, Murmansk, Novgorod, Vladimir, Orenburg, Voronezh, and a number of other regions [60, 62, 63]. According to O.A. Doshchannikova et al. (2018), the program aimed at supporting young professionals in rural areas helped to recruit more than 1,200 doctors in the Nizhny Novgorod Oblast between 2006 and 2017 [64].

In the Khanty-Mansiysk Autonomous Okrug – Yugra, measures of social support for people who arrived from other Russian regions and signed employment contracts include coverage of relocation costs for workers and their family members within the territory of the Russian Federation; paid vacation of seven calendar days for relocation; a one-time payment in the amount of two official salaries and an allowance

[1] Resolution of the Government of the Russian Federation (2022) On Amendments to Appendix 5 to the State Program of the Russian Federation "Development of Healthcare" No.739 of 22.04.2022.

[2] Resolution of the Government of the Russian Federation (2014) On establishing the order of providing social support measures for certain groups of individuals, who completed training programs in government-funded organizations specializing in post-graduate and residency programs No. 520 of 15.08.2014

for each family member; and reimbursement of rental costs for invited professionals in the range of 50 to 100% of the cost[1]. Furthermore, graduates of secondary and higher technical education institutions under the age of 30 who have signed an employment contract with a medical organization are entitled to a one-time payment in the amount of up to two monthly salaries.

Since 2017, the Orenburg Oblast has been using mentorship strategies to improve the level of skills of young experts throughout their first 12–24 months of employment. This approach involved 65 medical organizations, 1,470 young experts, and 740 mentors in 2017–2019.[2]

Similar strategies are implemented in the Ulyanovsk Oblast. Since 2013, over 2,000 healthcare workers have participated in the mentorship program, and the number of employed graduates climbed from 64 to 83% in 2016–2018[3].

Another important strategy implemented in the Russian Federation to recruit and retain young professionals is a stimulating monthly payment throughout the study in medical universities after signing an employer-sponsored training agreement. In the Sakhalin Oblast, for example, students are provided with coverage of extracurricular paid educational services, reimbursement of rental costs for accommodation throughout the study period, coverage of traveling costs to the place of internship and practical training in residency, and an additional payment throughout this period[4].

CONCLUSION

According to our review, there are several factors that influence the decision of healthcare professionals about rural practice, as well as their recruitment and retention in the rural area. All of the factors examined in this research are grouped into four categories: financial, social, professional, and personal. At the same time, these factors have different significance for various types of professionals. The most frequently described factors in the literature were financial ones: salary, scholarships and allowances throughout the training period, which were significant for young professionals. Personal factors, such as rural background, as well as education and practical training in rural areas, are of special interest. Numerous studies have demonstrated their relationship with retention and willingness to work in hard-to-reach areas. At the same time, more research is required to comprehend the characteristics of a future rural physician, including rural background, gender, character traits, and other factors, which will serve as the basis for selection and recruitment policy and pre-enrollment counseling for school leavers and senior high school students.

Currently, several strategies have been implemented to solve the problem of recruiting and retaining practitioners in rural areas – often a combination of various methods has been employed. The choice of specific measures should be based on a profound understanding of the characteristics of human resources in healthcare, which requires a comprehensive analysis of the labor market, staff distribution by the geographical area, and factors influencing the decision to stay in or leave rural areas.

According to the findings of the study, current methods may be divided into three types: financial, organizational, and educational. Based on the research results, the first, most effective, cost-effective, and affordable group of strategies includes educational measures. Based on international best practices, educational measures include practical training in rural areas to familiarize students with the specifics of work in rural medical facilities; decentralized training at rural medical facilities; work with school leavers living in rural areas as part of an admission campaign; placement of medical colleges and universities in rural areas; and development of career advancement programs that meet the needs of rural healthcare professionals.

The second set of measures includes financial benefits, such as scholarships for students who study in the employer-sponsored program, one-time payments for rural workers, and an increased salary coefficient. It is important for professionals to be able to choose

[1] Order of Healthcare Department of the Khanty-Mansiysk Autonomous Okrug – Yugra (2015) On approving draft Provisions on establishing remuneration for healthcare workers in medical facilities subordinate to the Healthcare Department of the Khanty-Mansiysk Autonomous Okrug – Yugra No. 13-np of 29.10.2015

[2] Resolution of the Ministry of Health of the Orenburg Oblast (2017) On approving provisions on mentorship programs in government-funded medical facilities No. 1949 of 07.09.2017

[3] Resolution of the Government of the Russian Federation (2019) On approving Provisions on mentorship in civil service in the Russian Federation No. 1296 of 07.10.2019

[4] Passport of the regional project "Staffing of medical facilities with highly qualified professionals (Sakhalin Oblast)"

one of the previously mentioned financial incentive strategies.

The third group includes organizational measures, such as an increase in job satisfaction due to improved working conditions in rural areas, flexible work schedules, improved living conditions (raising the physician's social status), opportunities for career growth, and creation of horizontal communications between urban and rural medical organizations.

Although current methods recognize multiple and interrelated effects of various factors, they are often challenging and do not allow for development of strategic actions. Furthermore, there is no assessment of the effectiveness of implemented measures in most countries, which hinders their distribution and transfer. Monitoring and assessing the implemented best practices are necessary to detect implementation restrictions, change policies if needed, learn important lessons, collect evidence, and deepen the understanding of how and why certain measures work in some circumstances but are ineffective in others. As a result, monitoring and assessment should be included into the implementation plan.

According to the findings of this review, the Russian Federation uses most of the global best practices for recruiting and retaining healthcare professionals in rural areas. However, some measures have yet to be implemented in our country, such as development of individual professional and career growth plans for respondents who are Doctors and Candidates of Sciences, integration of flexible work schedules for physicians of rare specialties, training at rural medical organizations, including decentralized training, and development and implementation of residency programs at rural medical organizations with field accreditation.

These best practices may be included in healthcare management practices to increase the effectiveness of regional programs for development of human capital in healthcare.

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Mysterious plastic bronchitis: a little-known disease in medical practice

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ABSTRACT

The article presents a clinical case of a 51-year-old patient first seeking medical care with complaints of paroxysmal cough bringing up bronchial casts. The diagnosis of plastic bronchitis was verified. The disease which has not been well described in the literature, difficulty of verifying the underlying diagnosis due to polysymptomatic clinical presentation characterized by the mortality rate of 50–80%, COVID-19 coinfection, resistance to therapy, and little concern of medical specialists determine the relevance and value of this clinical case.

Keywords: bronchial casts, plastic bronchitis, lymphatic plastic bronchitis, bronchial obstruction syndrome

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Загадочные «резиновые» слепки бронхов: малоизвестное заболевание в медицинской практике

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РЕЗЮМЕ

Представлено клиническое наблюдение пациентки, впервые обратившейся за медицинской помощью в возрасте 51 года по поводу приступообразного кашля с отхождением слепков бронхов. Верифицирован диагноз лимфопластического бронхита. Нозология, ранее не нашедшая подробного отражения в литературе, трудность верификации основного диагноза на фоне полисимптомной клинической картины, характеризующейся летальностью в 50–80%, сочетанное течение с новой коронавирусной инфекцией, а также резистентность к проводимой терапии и низкая настороженность медицинского сообщества определяют актуальность и ценность данного клинического наблюдения.

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Ключевые слова: слепки бронхов, пластический бронхит, лимфопластический бронхит, бронхообструктивный синдром

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Increased interest in respiratory pathology in recent years is explained by the pandemic of the SARS-CoV-2 coronavirus. However, bronchial obstruction syndrome in adults can also be caused by rare nosological entities, which include plastic bronchitis.

Plastic bronchitis (PB) (chronic fibrinous bronchitis, pseudomembranous bronchitis, mucoid impaction, Hoffman's bronchitis, lymphatic (lymphoid, lymphatic plastic) bronchitis) is a chronic recurrent bronchial inflammation, characterized by the formation of rubbery plugs (in the form of bronchial casts that are denser than normal mucous secretions) causing airway obstruction and respiratory failure [1–7].

The first descriptions of bronchial casts date back to the time of Galen (131–200 AD) who characterized them as "expectorated arteries and veins" [4, 8]. In 1750, Bussiere reported a postmortem examination of a patient suffering from tuberculosis. The patient was also found to have a bronchial cast *in situ* [4]. In 1902, M. Bettman described several cases of PB with bronchial casts and R. Shaw documented cases of mucoid impaction in 1951 [8]. By 1960, about 300 cases had been described in the literature [9, 10]. About 420 PB cases had been registered by 2008 [2, 10].

When searching for the term "plastic bronchitis" in the English-language text database PubMed in 2022, one could find 513 publications. Meanwhile, a detailed search for "adult plastic bronchitis" showed only one-fourth of these publications – 127. From 1965 to 2022, the number of manuscripts devoted to this topic increased, with the largest number of articles, reviews, and cases published in the last decade.

In our country, there are only few publications describing such clinical cases so far. Most of them, as well as in the foreign literature, are related to pediatrics. This can be explained by the peculiarities of the disease. It is diagnosed more often in children and at a young age. In adults, the disease is registered very rarely [1–6, 8, 9]. These cases in the absence of infectious and allergic genesis can be called casuistic. It is more common in women than in men [5]. At the same time, there is an opinion that it is slightly more frequent in men [4]. A higher prevalence is registered in dry and hot areas due to the changes in physical and chemical properties of airway secretions as a result of mucosal dehydration [10].

Unfortunately, there have been no large-scale epidemiological studies of PB in adults. The true incidence and prevalence rates cannot be accurately determined because it is likely that many episodes of the disease are left undiagnosed. The largest pool of observations of lymphatic PB in adults (n = 44) was published in 2022 by C. O'Leary et al. [11]. In Russia, the observational data of the largest cohort of PB patients (n = 20) for the period from 1990 to 2012 were published by V. Molodtsova et al. [5]. In 2021, Japanese scientists Y. Murata et al. described a case of the oldest patient. The 74-year-old woman had multiple myeloma and PB associated with a viral infection (influenza A virus) [11]. The problem is highly alarming not only due to underdiagnosis of this condition, but also because of a high mortality rate, which reaches 50–80%, according to some authors [4, 6, 7, 9].

The etiology and pathogenesis of the disease still remain completely uncertain (Table).

The clinical presentation of PB includes cough, dyspnea, crackles in the lungs, and cough bringing up bronchial casts. The course of the disease is complicated by atelectasis, bronchial obstruction syndrome, and fatal asphyxia [1, 3, 4].

Table

Etiology of plastic bronchitis*	
Etiology	Associated diseases
Primary and secondary lymphatic abnormalities	Congenital heart failure (heart defects) following surgical correction (Fontan, Glenn, Blalock – Taussig procedures). Chest trauma. Lymphatic abnormalities, lymphangiectasia, lymphangiomatosis
Viral infections	Influenza A virus, adenovirus infection, rhinovirus infection, respiratory syncytial virus, parainfluenza virus, SARS-CoV-2
Other lung diseases	Bronchial asthma, bronchopulmonary aspergillosis, bronchiectasis, cystic fibrosis
Bacterial infections	Streptococci, Haemophilus influenzae, Mycobacterium tuberculosis, Klebsiella, etc.
Hematological diseases	Acute respiratory distress syndrome in sickle cell anemia
Occupational diseases	Silicosis
Cancer	Kaposi's sarcoma with possible involvement of lymphatic vessels. Endobronchial metastases in kidney cancer

*Adapted from [4], [13].

The diagnosis of PB is confirmed by the presence of casts that have been coughed up or visualized during fibrobronchoscopy (FBS). A full clinical and laboratory examination is required for diagnosis [10]. The gold standard for verification and treatment of PB is dynamic contrast-enhanced magnetic resonance lymphangiography and intranodal lymphangiography, lymphatic embolization [10, 11] (Fig. 1).

CLINICAL CASE

Female patient V., 51 years old, born in Kogalym, is a doctor herself. The patient first sought medical help in April 2020, complained of paroxysmal cough bringing up bronchial casts. The casts had dense consistency (rubbery), white color (Fig. 2) and were coughed up mainly at night and in the morning, sometimes accompanied by abundant liquid sputum of milky white color. "Nagging" cough, "choking" with sputum at night, increased body temperature, shortness of breath, and difficulty exhaling during normal physical activity.

The patient became acutely ill in March 2020, when the clinical symptoms of acute respiratory viral infection appeared (body temperature increased to 38 ºC, runny nose, paroxysmal cough). The therapy (with antiviral drugs, expectorants, mucolytics) resulted in few positive changes: cough improved, body temperature lowered, rhinorrhea disappeared.

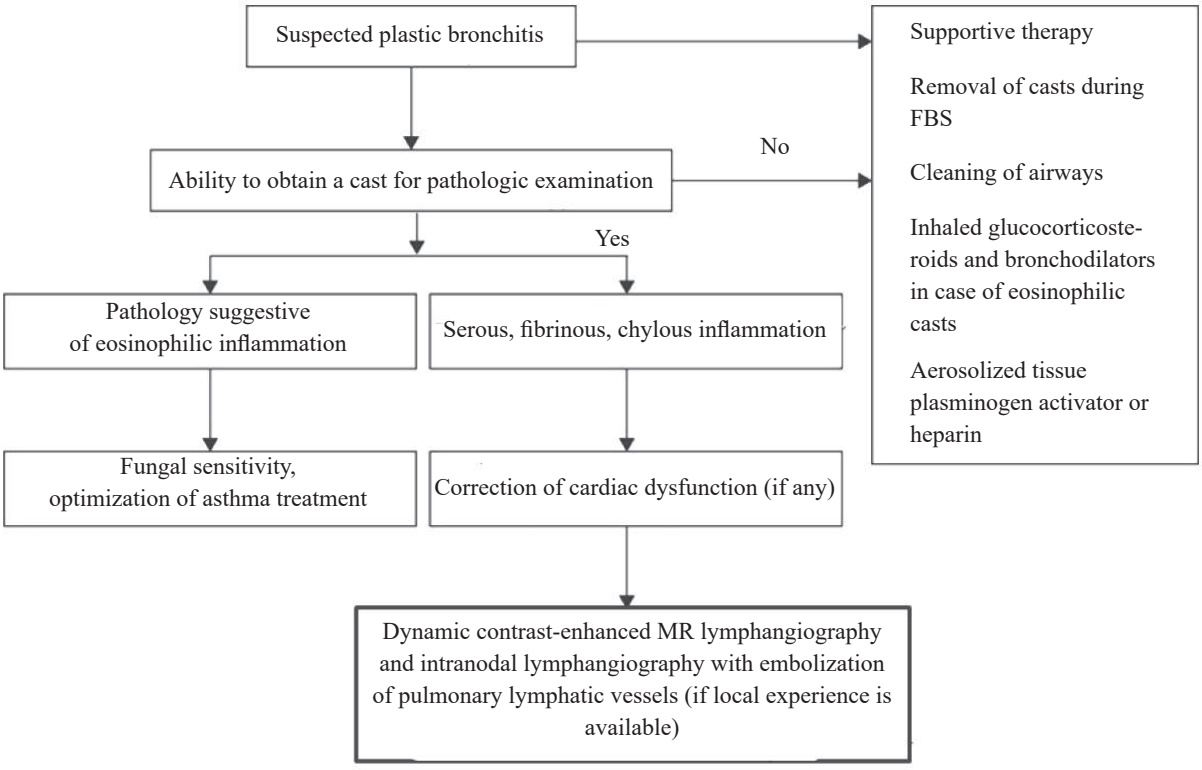


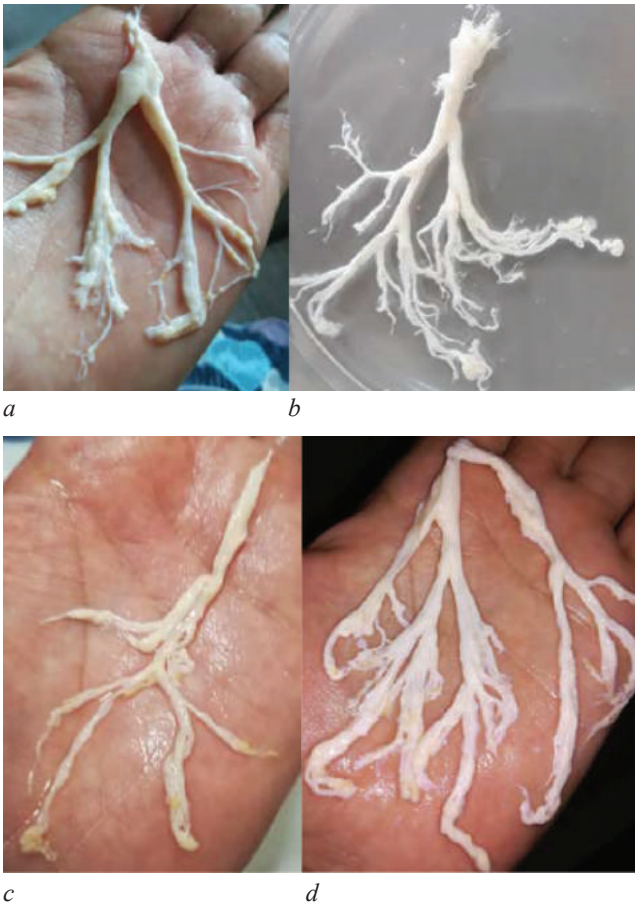
Fig. 1. Algorithm of diagnosis and management of PB patients [4]

Two weeks later, the patient first complained of abundant expectoration of milky-white sputum. For the first time, she coughed up small rubbery bronchial casts. Past medical history: hypertension and type 2 diabetes mellitus verified 5 years ago. In 2010, she underwent left mastectomy for breast cancer. Objective examination findings: postoperative scar in the area of the left breast, no abnormality detected, body mass index 33.3 kg / m^2, rough breathing on the left side in the lower parts of the lungs, and oxygen saturation of 96%.

Based on the available data, the patient was diagnosed with acute bronchitis. The antibacterial therapy that was prescribed did not have any effect. A month after a contact with a COVID-19 patient, a woman reported that her condition aggravated: body temperature increased to 39ºC, cough became stronger, the volume of sputum increased, dyspnea appeared. The patient was diagnosed with COVID-19. Doctors made a differential diagnosis with pneumonias of different infectious genesis, tuberculosis, and allergic lung damage. The FBS findings were remarkable: on the left, the upper lobe bronchus orifice was obturated with cheesy necrotic mass, mucosa was hyperemic, the lumen contained a moderate amount of turbid sputum. The patient received treatment according to the temporary clinical guidelines for COVID-19, as well as symptomatic therapy. During the FBS, the patient's necrotic tissue was removed and airways were cleared. After discharge, the patient's condition rapidly deteriorated: dyspnea became worse, cough became hacking and more intense, the volume of expectorated sputum increased. The patient started to cough up bronchial casts again at night and in the morning (Fig. 2).

Each paroxysm ended with coughing up a cast. Due to the deterioration of symptoms, the patient was referred to the Ural Research Institute of Phthisiopulmonology (23.06.2022–13.07.2022).

Lung computed tomography (CT) revealed bilateral polysegmental lung lesion in the regression phase, left lung lesion – ground glass opacity, reactive lymphadenopathy of intrathoracic lymph nodes. A cytological examination of the casts was performed: mucus clumps with groups of bronchial epithelial cells having reactive cellular changes, few macrophages with leukocytes (bronchial casts), admixture of traumatic tap blood. There was no data indicating tumor growth (Fig. 3). The available data suggested the diagnosis of PB.



a *b* *c* *d*

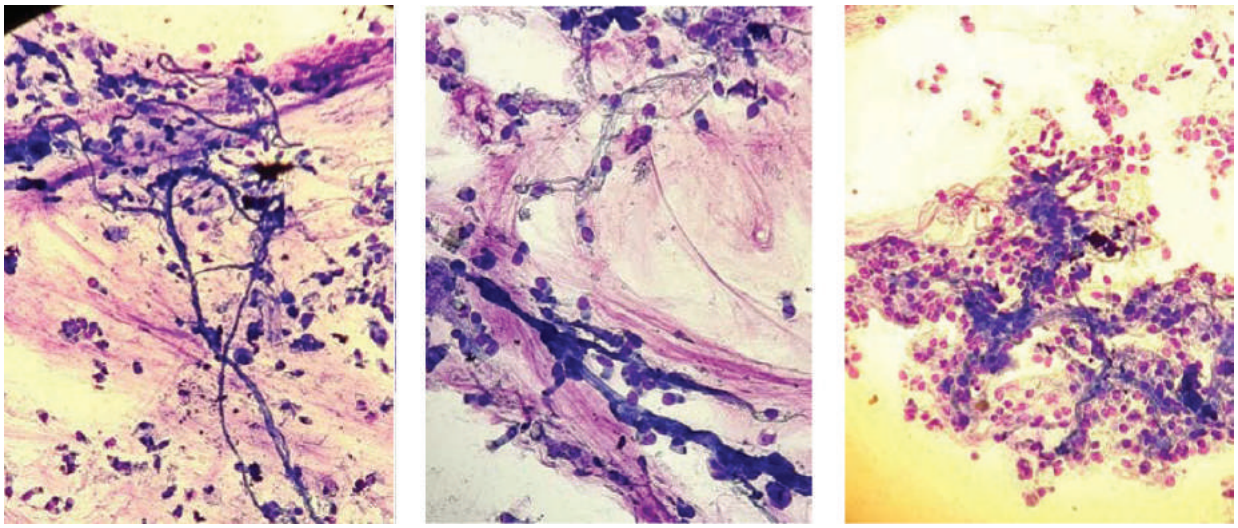
Fig.2. Patient V., 51 years old. Sputum in the form of bronchial casts

A woman was consulted by V. Parshin, thoracic surgeon, Doctor of Medical Sciences, Professor, corresponding member of the Russian Academy of Sciences, Head of the Surgical Department of the I.M. Sechenov Clinical Center. The patient was also remotely consulted by Maxim Itkin, thoracic surgeon, Professor of Radiology at the Hospital of the University of Pennsylvania, Director of the Center for Lymphatic Imaging and Interventions (Philadelphia, USA). Based on the features that can be considered the disease criteria, which are: ground glass opacity of one lung according to lung CT, sputum in the form of bronchial casts, lymphadenopathy of the intrathoracic bronchial nodes, obesity, increased expectoration of sputum after eating fatty foods, absence of convincing data about an allergic process, PB of suspected lymphatic genesis was confirmed. The detection of neutral fat in bronchial lavage fluid would have been an additional criterion, but this procedure was not carried out.

In February 2021, doctors performed ligation of the thoracic duct above the diaphragm and transection of

the lymphatic vessels of the left lung root according to the root skeletonization method at the Department of Thoracic Surgery of the Sechenov University Clinical Hospital No.1.

Thirty minutes prior to the surgical intervention, the patient was offered to drink 200 ml of a fatty mixture (sour cream (20%), cream (10%), and butter) to detect a lymphatic vessel leakage. After that, the chest was opened and a lymphatic vessel causing abundant lymphorrhea was found. A cyst of the thoracic lymphatic duct was also detected intraoperatively. In the early postoperative period, the patient started to adhere to an oral diet of fat-free food gradually extending a range of food products. Final diagnosis: thoracic duct cyst (parabronchial, collateral cyst on the left). Lymphatic plastic bronchitis (chylobronchorrhea). Lymphadenopathy of the mediastinum. Recommendations: low-fat diet (1 month), expanding meals after 1–1.5 months, restriction of physical activity for 2 months, correction of body weight. One year later, the woman had no complaints, the above symptoms were eliminated completely, the patient did not follow the diet. According to the lung CT (April 2022), the patient still had pulmonary fibrosis of the left lung.



a *b* *c*

Fig. 3. Cytological examination of bronchial casts of patient V

DISCUSSION

Difficulties making the diagnosis in this case were associated with the clinical polymorphism of the disease, low awareness of specialists about this pathology, COVID-19 coinfection, which could have both initiated and masked the disease, and the lack of convincing data during the examination for a lymphatic vessel defect causing the patient's symptoms.

The true etiology of the pathological changes in the observed woman remains an open question. Several causes and factors may be involved. In recent years, some scientists have supposed that abnormal communication and leakage of lymphatic fluid into the airway are some of the main PB causes. Lymphatic duct obstruction can explain PB in cardiac surgery, heart failure or trauma [9, 12]. Many cases of idiopathic PB in adults are associated with lymph, which allows us to rename the diagnosis in these patients into "lymphatic plastic bronchitis (lymphatic PB)" [7, 11]. It is possible that the left mastectomy performed 12 years ago caused pathological changes in this patient. Increased pressure in the lymphatic vessels observed in congenital lymphangiectasia or lymphangiomatosis can lead to retrograde lymph flow, drainage of lymph into the bronchial lumen, and chylothorax development [13].

CONCLUSION

The disease has not been well described in the literature. It is difficult to diagnose due to the poly-symptomatic clinical presentation and manifestations that coincide with different bronchopulmonary pathologies. The disease is characterized by the mortality rate of 50–80%, COVID-19 coinfection, resistance to therapy, and low awareness of medical

specialists about it. These reasons determine the relevance and value of this clinical case.

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A clinical case of co-occurring mental disorder and coronavirus infection

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ABSTRACT

In the new millennium, humanity has faced with a global challenge in the form of the novel coronavirus infection (COVID-19). In addition to systemic and respiratory symptoms, SARS-CoV-2 causes neurological disorders, as it is a neurotropic virus. Many scientists assume that SARS-CoV-2 can enter the nervous system through the functional receptor of angiotensin-converting enzyme 2, which is present in glial cells, neurons, skeletal muscles, and other organs. Neurological complications are manifested by damage to the central nervous system, peripheral nervous system, and cranial nerves, as well as by mental disorders. Mental illnesses develop due to neuroinflammation and neuronal death after brain infection with SARS-CoV-2.

The article describes a clinical case of a 63-year-old man with the co-occurring novel coronavirus infection and obvious mental disorder who has never had any mental illnesses before. The given clinical example demonstrates the importance of studying the cause-and-effect relationship between COVID-19 and mental illness. In the medium- and long-term perspective, COVID-19 is expected to result in mental health disorders during COVID-19 recovery. Besides, an increase in the number of patients with mental disorders who were mentally healthy before COVID-19 infection is also expected.

Keywords: SARS-COV-2, COVID-19, coronavirus, mental illnesses, mental disorders

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Клинический случай психического расстройства на фоне новой коронавирусной инфекции

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РЕЗЮМЕ

В новом тысячелетии человечество столкнулось с глобальной проблемой – новой коронавирусной инфекции COVID-19, вызванной вирусом SARS-CoV-2. Помимо системных и респираторных симптомов, вирус SARS-CoV-2 вызывает неврологические расстройства, так как обладает нейротропностью. Многие ученые предполагают, что SARS-CoV-2 способен проникать в нервную систему через функциональный рецептор ангиотензин-превращающего фермента 2, который присутствует в глиальных клетках, нейронах, скелетных мышцах и других органах. Осложнения со стороны нервной системы проявлялись поражениями различных структур: центральной нервной системы, периферической нервной системы и черепно-мозговых нервов, а также психическими расстройствами. Психические заболевания развиваются вследствие нейровоспаления и гибели нейронов после заражения мозга SARS-CoV-2.

Описан клинический случай новой коронавирусной инфекции у мужчины 63 лет с впервые выявленным психическим расстройством. Приведенный клинический пример демонстрирует важность изучения причинно-следственной связи между COVID-19 и психическим заболеванием. В среднесрочной и долгосрочной перспективе ожидается, что COVID-19 приведет к проблемам психического здоровья в период постковидного восстановления, также ожидается увеличение количества пациентов с психическими расстройствами, которые были психически здоровы до заражения COVID-19.

Ключевые слова: SARS-COV-2, COVID-19, коронавирус, психические заболевания, психические расстройства

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

The coronavirus infection (COVID-19) is a global public health emergency which has a great impact on mental health. To date, it has been determined that COVID-19 affects not only the lungs, but also cells of the central nervous system. Data on the SARS-CoV-2 neuroinvasion [1–3] and neuropsychiatric consequences of the disease caused by it are increasing [4]. It is known that 0.9–4% of infected people develop psychotic spectrum disorders. The link between COVID-19 and mental illness has been established by many observational studies, but a causal relationship cannot yet be reliably detected.

M. Taquet et al. [5] found that the first psychiatric diagnosis is more common in patients with COVID-19 within 14–90 days after the first COVID-19 symptoms, and a psychiatric diagnosis may be an independent risk factor for COVID-19. The scientists expand their findings by assessing the incidence rates and relative risks of 14 neurological and psychiatric diagnoses in patients within 6 months after COVID-19 was diagnosed. Cerebral edema and partial neurodegeneration were described in post-mortem reports, which indicates that the virus might contribute to the development of acute psychiatric symptoms and long-term psychoneurological consequences of COVID-19.

Neuropsychiatric conditions are accompanied by profound changes in the morphology and function of microglia, leading to the secretion of proinflammatory cytokines. Meanwhile, aberrant phagocytosis affects neural circuits. Conceptually, long-term disruption of neuroglial function ultimately affects synaptic integrity, the excitation – inhibition balance, and information processing, making a fundamental contribution to the pathogenesis of neuropsychiatric disorders. J. Rogers et al. suggest that emerging neuropsychiatric manifestations may be due to brain exposure to the virus, indirect immune responses or ongoing treatment [6]. However, mental disorders developing at the acute stage of the disease may arise due to fears about the consequences of the disease, such

as social isolation and quarantine [7], unemployment, financial difficulties [8], and stigma [9].

CLINICAL CASE

Patient A., 63 years old, was admitted to a repurposed inpatient hospital for infectious diseases in Aktobe in April 2021. The patient complained of dyspnea on exertion, cough, insomnia, lower quality of sleep, absence of taste and smell, fever up to 39˚C, and severe generalized weakness.

According to the medical history, the patient was diagnosed with hypertension and coronary heart disease. The patient regularly takes lisinopril 5 mg, atorvastatin 20 mg, acetylsalicylic acid + magnesium hydroxide 75 mg. Bad habits include smoking for 35 years, half a pack a day.

From the anamnesis vitae: no mental illnesses in the family history. The patient grew and developed according to his age.

The objective examination findings: severe general condition, lucid, normal physique. Body temperature is up to 38–39 ºC. The pharynx is hyperemic, moist. The heart rate is 97 beats per minute (tachycardia), the blood pressure is 160 / 90 mm Hg. Saturation level is 91% in room air. A nasopharyngeal PCR swab for SARS-CoV-2 is positive. Breathing is weakened in the lower parts of the lungs, small bubbling and moist rales are heard on auscultation. The respiratory rate is 22–24 breaths per minute.

The laboratory tests: C-reactive protein – 80 mg / l (normal range < 10 mg / l), D-dimer – 980 mg / l (< 250 mg / l), ferritin – 216 µg / ml, interleukin (IL) – 11.6 pg / ml, other inflammatory markers were within the normal range. The complete blood count: lymphopenia – 9%, thrombocytopenia – 156 / l, leukocytosis – 19 / l, creatinine – 157.0 µmol / l, urea – 15 mmol / l. Chest X-ray revealed bilateral polysegmental pneumonia.

The mental state of the patient at admission was characterized by fussiness, anxiety, and motor restlessness. The patient was admitted to the infectious disease hospital on the 11th day from the disease onset. On the 4th day of hospital stay, due to a change in his mental state characterized by agitation, aggressive behavior, and insomnia, the patient was examined by a psychiatrist and a neurologist. During the examination, the patient complained of insomnia, anxiety, and fear for his life, repeated that “everyone will die from the coronavirus, the Chinese are to blame, they want to kill us, Nazarbayev and Tokayev betrayed me, they made a mistake, they committed a sin, all of us are in danger”. When asked by doctors, he said that “death has come, we must face it with dignity”, and asked the neighbors in the ward to bathe and dress in clean clothes. During the 4 days of hospital stay, he became uncommunicative, secluded himself, behaved suspiciously, declared that the end of the world would come soon, “the spirit of the deceased brother wants to take his soul”. In the last two nights, he did not sleep, it seemed to him that “the devil came to take his soul”. He started calling the Ministry of Emergency Situations and President Vladimir Putin using a mobile phone.

The mental status: lucid, untidy looks, contactable, tense, suspicious, anxious, without a sense of distance, speech in the form of a spontaneous monologue. During the conversation, he jumped up from his chair, went out into the corridor, came to the windows, then returned and said: “The Chinese are to blame, they want to kill us, Nazarbayev and Tokayev betrayed me, they made a mistake, they committed a sin, all of us are in danger”. He did not reveal painful experiences, stayed in the world of worries and concerns. He looked around, listened, sometimes smiled to himself. Thinking was inconsistent, paralogical, accelerated. His behavior was characterized by motor restlessness and impulsiveness with signs of severe emotional instability. Intellectual mnestic functions were not greatly affected despite the limited range of interests. The clinical presentation of acute psychosis was dominated by a severe affective delusional disorder and the inability to critically assess his condition. The patient was diagnosed with organic delusional disorder.

The neurological status: no focal neurological symptoms were detected.

CONCLUSION

Therefore, some of the neurological complications of COVID-19 are mental disorders. Patients with a concomitant pathology are at risk for severe COVID-19. Severe hypoxia and hypoxic encephalopathy are manifested by diffuse brain damage and death of its cells. Some patients may develop severe delirium, others – encephalopathy, drowsiness, and impaired consciousness.

This patient had an increased immune response. High levels of IL-6 indicate a decrease in body immunocompetence. The host immune response to the SARS-CoV-2 infection and possible direct viral infections of the central nervous system are potential mechanisms that can cause neuropsychiatric

consequences [10]. Proinflammatory cytokines are known to cause axon degeneration by changing the activity of neurons and glia. That will change the mental status and lead to neurocognitive disorders, headache, encephalitis, myelitis, stroke, myopathy, Guillain – Barré syndrome and polyneuropathies [11]. Systemic inflammation exacerbates cytokine-mediated brain damage and hematogenous spread of SARS-CoV-2 into the brain [12].

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Best practices in the use of human immunoglobulin preparations for intravenous administration in the treatment of rare neurological diseases

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ABSTRACT

Aim. To describe best practices in using human normal immunoglobulin in patients with immune-mediated neurological disorders according to the data of one clinical center.

Materials and methods. From 2016 to 2021, 20 patients with various autoimmune disorders of the peripheral and central nervous system were treated with human normal immunoglobulin at the Neurology Unit No.1 of Pavlov First Saint Petersburg State Medical University. Treatment efficacy was assessed by changes in the neurological examination data according to specialized scales for specific diseases or clinical manifestations (INCAT, QMGS, MoCA, EDSS). Safety of the therapy was assessed considering the instructions to the drug.

Results. In the vast majority of patients, treatment allowed to stabilize the course of the disease or was accompanied by pronounced regression.

Conclusion. The considered clinical cases of the use of human normal immunoglobulin preparations demonstrate the possibility of their use in the treatment of a number of autoimmune neurological diseases for unregistered indications.

Keywords: human normal immunoglobulin, myasthenia gravis, chronic inflammatory demyelinating polyneuropathy, multifocal motor neuropathy, autoimmune encephalitis, Guillain – Barré syndrome, immunotherapy, intravenous administration

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Опыт применения препаратов иммуноглобулина человека для внутривенного введения в лечении редких неврологических заболеваний

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РЕЗЮМЕ

Целью работы является обобщение опыта применения иммуноглобулина человека нормального у пациентов с имуноопосредованными неврологическими заболеваниями по данным одного клинического центра.

Материалы и методы. С 2016 по 2021 г. на базе неврологического отделения № 1 ПСПбГМУ им. И.П. Павлова проведено лечение иммуноглобулином человека нормальным 20 пациентов с различными аутоиммунными неврологическими заболеваниями периферической и центральной нервной системы. Оценку эффективности лечения проводили, анализируя динамику данных неврологического осмотра в соответствии со специализированными для конкретных заболеваний или клинических проявлений шкалами (INCAT, QMGS, MoCA, EDSS). Безопасность терапии оценивали с учетом инструкции по применению лекарственного препарата.

Результаты. У подавляющего числа пациентов лечение позволило стабилизировать течение болезни или сопровождалось выраженным регрессом симптомов.

Заключение. Рассмотренные клинические случаи применения препаратов иммуноглобулина человека нормального демонстрируют возможность их использования в лечении ряда аутоиммунных неврологических заболеваний по незарегистрированным показаниям.

Ключевые слова: иммуноглобулин человека нормальный, миастения, острая и хроническая воспалительная демиелинизирующая полинейропатия, мультифокальная моторная нейропатия, аутоиммунный энцефалит, синдром Гийена – Барре, иммунотерапия, внутривенное введение

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

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INTRODUCTION

Currently, for the treatment of a wide range of immune-mediated diseases of the central and peripheral nervous system (Guillain – Barré syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), autoimmune encephalitis, etc.), drugs with various patented names and a single international non-proprietary name “human normal immunoglobulin” are used. The dosage forms of these drugs are intended primarily for intravenous administration and are often referred to as “intravenous immunoglobulins” (IVIG).

IVIG preparations contain human multispecific immunoglobulins (Ig), predominantly of class G, obtained from the blood plasma of healthy donors, which contain antibodies (AB) to a variety of antigens, given a high level of viral safety is ensured [1, 2]. They may differ in the ratio of Ig A and Ig G and in the mechanisms of antigen inactivation. For the maximum therapeutic effect, the drug must contain at least 95% Ig G with distribution in subclasses 1–4 comparable to normal serum and with a minimum amount of Ig A [2, 3].

IVIGs are used not only in replacement therapy for various immunodeficiencies, but also as immunomodulatory agents; however, the mechanism of their pharmacological and biological effects is not fully understood. Depending on the predominant participation of Fab- (fragment antigen binding) or Fc- (fragment crystallizable) fragments of immunoglobulin in the reactions, two possible mechanisms are considered [1, 3].

The first group includes Fab-mediated neutralization of autoantibodies, proinflammatory cytokines, and activated complement components, induction of apoptosis, inhibition of leukocyte adhesion molecules, and restoration of the idiotypic – anti-idiotypic network. It is believed that anti-idiotypic AB can bind auto-AB, for example, to Nm acetylcholine receptors in myasthenia gravis or to gangliosides in Guillain – Barré syndrome [3]. The second probable mechanism, involving the Fc fragment, includes activation or inhibition of macrophages, monocytes, and dendritic cells, as well as suppression of autoreactive B cells and interaction with natural killers [3]. The listed effects partly develop already in the process of

infusion, however, a clinically significant effect after IVIG administration is usually recorded after the second day and lasts about 3–5 weeks, depending on the half-life of a particular drug, which is determined both by the properties of the molecule and, in some cases, by factors of the body. It is the pharmacokinetic parameters that determine the average duration of the IVIG course, which ranges from 3 to 5 days [3]. According to studies, IVIG drugs have an anti-inflammatory effect at high doses, while at low doses they can have an undesirable proinflammatory effect [1]. When choosing an IVIG preparation for treatment of autoimmune neurological diseases, the absence of restrictions on single and daily doses, the maximum allowable rate of administration, and the stabilizer used in the manufacturing are very important [2]. According to the current guidelines in the Russian Federation (https://grls.rosminzdrav.ru), indications for the use of IVIGs in neurology are limited to Guillain – Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), and multifocal motor neuropathy (MMN) (Table 1).

Table 1

Human normal immunoglobulin preparations for intravenous administration registered in the Russian Federation for the treatment of neurological diseases (State Register of Medicines, 09.02.2021)

Disease	Trade name of IVIG *
Guillain – Barré syndrome	Kiovig, Immunoglobulin Sigardis, Immunoglobulin Sigardis MT, Privigen, Octagam, Intratect, Octagam 10%, Gabreglobine-IgG, Flebogamma 5% DIF, I.G.Vena, Gamunex-C
Chronic inflammatory demyelinating polyneuropathy	Privigen, Intratect, Gamunex-C
Multifocal motor neuropathy	Kiovig, Intratect

Note: according to the instructions to the drug, Pentaglobin, Imbioglobulin, Immunovenin, Gabreglobine, and Immunoglobulin human normal (manufactured by Microgen and Nizhny Novgorod Regional Blood Center named after N.Ya. Klimova) intended for intravenous administration can only be used for replacement therapy for immunodeficiencies.

Despite the fact that in some cases the use of IVIGs is optimal in terms of safety and efficacy, there is a fundamental problem in the form of the absence of indications for some rare neurological disorders in the instruction to the drug. Human normal immunoglobulin preparations are widely used in international practice for treatment for myasthenia gravis (MG), idiopathic inflammatory myopathies, stiff person syndrome, CIDP associated with monoclonal gammopathy of undetermined significance (MGUS), some forms of epilepsy, and active multiple sclerosis in women during pregnancy. In these diseases, off-label use of IVIGs takes place. At the same time, they are included in the standards of medical care for CIDP, MG, and acute disseminated encephalomyelitis (ADEM) [3, 4]. The aim of this work was to describe best practices in using human normal immunoglobulin in patients with immune-mediated neurological disorders according to the data of the Neurology Department No.1 at Pavlov First Saint Petersburg State Medical University.

MATERIALS AND METHODS

A monocenter, observational, retrospective and prospective cohort study on the efficacy and safety of IVIG in the treatment of immune-mediated neurological diseases was conducted on the basis of the neurological department No.1 of Pavlov First Saint Petersburg State Medical University in 2016–2021. The study included 20 people (14 women and 6 men), median onset of the disease was at the age of 49.5 [35.3; 58.5] years. Of these, 3 patients were diagnosed with MMN, 9 – with CIDP, 3 – with MG, 2 with autoimmune encephalitis, 2 – with neuromyelitis optica spectrum disorders (NMOSD), 1 – with stiff person syndrome (Table 2). IVIG preparations were used following medical indications according to the instructions or by the decision of an expert board of physicians in cases of off-label use when assessing the risk – benefit ratio (according to the order of the Ministry of Health and Social Development of the Russian Federation No. 494 of 09.08.2005 "On the procedure for using drugs in patients for health reasons" and the Federal Law No. 323-FZ of 21.11.2011). The therapy used human normal immunoglobulin preparations of various trade names.

All patients signed an informed consent to IVIG treatment. Medicines were administered intravenously by trained medical staff directly in the patient's ward. The procedure was carried out in accordance with the instructions and clinical recommendations or according to the data of expert working groups in the case of off-label application. Prior to infusion, adequate hydration was performed, hematocrit, IgA, and creatinine levels were determined [2], and thromboembolism risks were assessed. In order to prevent the development of systemic post-infusion reactions, premedication with paracetamol (1,000 mg, orally) and chloropyramine (20 mg, intramuscularly) was performed in all cases [5]. During the infusion and within an hour after its completion, the patient was under the supervision of a physician assessing the somatic status.

The dynamics of the neurological status was assessed daily using scales developed for the respective diseases (INCAT, QMGS, MoCA, EDSS). Safety of IVIG therapy was assessed by the presence and severity of adverse events [5]. Follow-up from the initiation of the therapy ranged from 2 to 30 months.

Statistical processing of the obtained results was not carried out due to the small sample size and the heterogeneity of the data.

RESULTS

The main characteristics of the patients, as well as regimens and results of IVIG therapy are presented in Table 2.

Among patients with CIDP, the phenotype of sensorymotor neuropathy was present in 7 patients, predominantly motor phenotype – in 2 individuals. In 4 cases, a progressive course of polyneuropathy was observed, in 5 – recurrent. Two patients (22%) with CIDP after two courses of IVIG therapy according to the INCAT scale showed partial regression of symptoms. Five patients (56%) had stabilization and no progression of symptoms after two courses of therapy with IVIG at a dose of 2 g / kg. In four (80%) out of five patients with a recurrent course, new exacerbations were prevented during the entire follow-up period.

In a patient with CIDP associated with MGUS, within 7 months of continuous monthly IVIG therapy with a starting dose of 2 g / kg (134 g within 5 days) in the first month and subsequent monthly course doses of 1 g / kg (67 g within 3 days), complete clinical remission was observed. A patient with an atypical variant of CIDP and concomitant podocytopathy after a course of therapy at a total dose of 2 g / kg developed an exacerbation 5 days after the end of the therapy.

In the case of a patient with stiff person syndrome, after 7 courses of regular IVIG therapy (at a dose of 1–2 g / kg), a clinical improvement was observed in the form of a decrease in axial and limb hypertonicity, as well as a decrease in the severity of dysarthria.

In 2 patients with autoimmune encephalitis associated with antibodies to GAD, after 6 and 12 months of treatment, respectively, stabilization of the condition, no progression of cognitive deficit, and regression of epileptic seizures were observed.

In a patient with an exacerbation of NMOSD in the second trimester of pregnancy and a clinical presentation of complete transverse myelitis at the cervical level, after a course of IVIG, significant regression of symptoms was noted with changes in the EDSS score from 4.0 to 2.5. Against the background of subsequent administration of the drug at maintenance doses for 2 months, no exacerbations were observed during the six-month follow-up.

A patient with NMOSD associated with SLE and severe lymphopenia underwent IVIG anti-relapse therapy for 3 months monthly, at a starting dose of 2 g / kg and a maintenance dose of 0.4 g / kg until the level of lymphocytes was restored. No exacerbations and negative trend in the EDSS score were registered during the follow-up. Subsequently, the therapy was shifted to rituximab.

All patients with stage IVb and V MG according to MGFA showed partial regression of symptoms and an improvement by 13 points according to the QMGS scale. Tolerability of IVIG therapy was assessed as good. No infusion reactions were reported in any of the cases. No delayed adverse events were observed during the follow-up period.

DISCUSSION

The results of our observational study indicate that long-term course use of IVIG preparations in a heterogeneous group of neurological patients in most cases leads to stabilization of the disease course and regression of symptoms. Immunoglobulin was effective and safe not only in cases of its use according to indications, but also in off-label application, due to a lack of other optimal or approved treatment options for severe diseases of the peripheral and central nervous system. The starting and maintenance doses were determined by the instructions to the drug or clinical guidelines and expert working groups in the case of off-label use. The duration of therapy and follow-up varied, from 5 days to 2.5 years, depending on the disease and clinical situation.

Our positive experience with the use of IVIG in the treatment of a number of autoimmune neurological diseases confirms the data obtained by other authors [2–4]. It is known that IVIGs are approved for the treatment of a number of diseases and are a first-line therapy in emergency neurological practice. For example, in patients with GBS, IVIGs are effectively used at a dose of 0.4 g / kg / day for a course of 3 to 5 days (up to 2 g /kg per course) [3, 4]. There were no patients with GBS among the participants in our observational study, and, therefore, a comparative assessment of the effectiveness of IVIG in this pathology was not carried out.

Table 2

Clinical and demographic characteristics of patients receiving IVIGs and features of the therapy

No.	Sex/age of disease onset/initiation of IVIG therapy (years)	Diagnosis	Therapy before IVIG administration	Course dose, number of courses (1 course – 5 days, monthly)	Duration of follow-up since the initiation of therapy	Dynamics of the patient's condition during follow-up	Assessment using standard scales	
							Before treatment	After treatment
1	F/47/55 years	CIDP	GCS	2 g/ kg, 1 course	2 months	E -, P -, PR	INCAT for arms 3, for legs 3	INCAT for arms 3, for legs 3
2	M/50/51 years	CIDP	GCS	2 g/ kg, 2 courses	4 months	E -, P -, PR	INCAT for arms 4, for legs 2	INCAT for arms 2, for legs 2
3	F/60/60 years	CIDP	GCS, plasmapheresis	2 g/ kg, 2 courses	4 months	E -, P -, PR	INCAT for arms 4, for legs 3	INCAT for arms 1, for legs 0
4	M/58/59 years	CIDP	GCS	2 g/ kg, 1 course	2 months	E -, P -, PR	INCAT for arms 1, for legs 2	INCAT for arms 1, for legs 2
5	F/58/58 years	CIDP	Did not have	2 g/ kg, 2 courses	2 months	E -, P -, PR	INCAT for arms 3, for legs 2	INCAT for arms 3, for legs 2
6	M/27/27 years	CIDP	GCS	2 g/ kg, 1 course	2 months	E -, P -, PR	INCAT for arms 4, for legs 3	INCAT for arms 4, for legs 3
7	M/64/65 years	CIDP	GCS, plasmapheresis	2 g/ kg, 1 course	2 months	E -, P -, PR	INCAT for arms 3, for legs 1	INCAT for arms 3, for legs 1
8	F/33/34 years	CIDP +MGUS	Plasmapheresis	2 g/ kg, 1 course 1 g/ kg, 6 courses	7 months	E -, P -, CR	INCAT for arms 1, for legs 1	INCAT for arms 0, for legs 0
9	M/56/56 years	CIDP + podocytopathy	GCS, plasmapheresis	2 g/ kg, 1 course	4 months	E +, P +, PR	INCAT for arms 4, for legs 5	INCAT for arms 4, for legs 5
10	F/67/69 years	MMN	Did not have	2 g/ kg 1 g/ kg 2 courses	4 months	E -, P -, PR	INCAT for arms 3, for legs 0	INCAT for arms 3, for legs 0
11	F/38/41 years	MMN	Did not have	2 g/ kg 24 courses	30 months	E -, P -, CR	INCAT for arms 4, for legs 0	INCAT for arms 1, for legs 0
12	M/52/56 years	MMN	Did not have	2 g/ kg 1 course	2 months	E -, P -, PR	INCAT for arms 3, for legs 0	INCAT for arms 2, for legs 0
13	F/49/50 years	Stiff person syndrome	GCS	2 g/ kg, 3 courses 1 g/ kg, 4 courses	11 months	E -, P -, PR	*	*

14	F/30/30 years	AE, GAD +	GCS, Rituximab	2 g/ kg, 1 course 0,4 g/ kg, 6 courses	12 months	E -, P -, PR	**MoCA 11	**MoCA 11
15	F/37/40 years	AE, GAD +	Did not have	2 g/ kg, 5 courses	6 months	E -, P -, PR	***	***
16	F/26/30 years	NMOSD, AQP4-IgG+, Exacerbation, 23 weeks pregnant	Did not have	2 g/ kg 0.4 g/ kg 0.4 g/ kg 3 courses	6 months	E -, P -, PR	EDSS 4.0	EDSS 2.5
17	F/36/37 years	NMOSD AQP 4-IgG+, SLE, immunodeficiency	GCS, Rituximab	2 g/ kg 0.4 g/ kg 0.4 g/ kg 3 courses	4 months	E -, P -, PR	EDSS 1.5	EDSS 1.5
18	F/21/25 years	MG, exacerbation	GCS, Azathioprine, plasmapheresis	2 g/ kg 1 g/ kg 1 g/ kg 1 g/ kg 4 courses	4 months	E -, P -, PR	QMGS 26	QMGS 13
19	F/67/68 years	MG, exacerbation	GCS	2 g/ kg 1 course	2 months	E -, P -, PR	QMGS 23	QMGS 10
20	F/63/64 years	MG, exacerbation	GCS, Azathioprine, plasmapheresis	2 g/ kg 1 course	2 months	E -, P -, PR	QMGS 26	QMGS 13

Note: AE – autoimmune encephalitis; GCS – glucocorticosteroids; F – female; NMOSD – neuromyelitis optica spectrum disorders; M – male; MG – myasthenia gravis; MGUS – monoclonal gammopathy of undetermined significance; MMN – multifocal motor neuropathy; E – exacerbation, yes (+) / no (-); P – progression, yes (+) / no (-); CR / PR – complete / partial regression of symptoms; SLE – systemic lupus erythematosus; CIDP – chronic inflammatory demyelinating polyneuropathy; AQP4-IgG – antibodies to aquaporin 4; EDSS – Expanded Disability Status Scale; GAD – anti-glutamic acid decarboxylase antibodies; INCAT – Inflammatory Neuropathy Cause and Treatment Disability Score; MoCA – Montreal Cognitive Assessment; QMGS – Quantitative Myasthenia Gravis Score.

* There are no validated symptom scores for stiff person syndrome; against the background of the therapy, there was a decrease in the severity of stiffness.

** There are no validated symptom scores for autoimmune encephalitis. The patient's clinical presentation was dominated by cognitive impairment, thus, the MoCA scale was used.

*** The clinical presentation was dominated by generalized tonic – clonic seizures; there are no validated symptom scores for autoimmune encephalitis; against the background of the therapy, there was a decrease in the frequency of seizures.

IVIG is used as a drug of choice in the treatment of CIDP, including CIDP associated with diabetes mellitus and uncontrolled arterial hypertension, as well as in an atypical course of the disease, including motor phenotypes [6–8]. The generally accepted starting dose is 0.4 g / kg / day, from 3 to 5 days (up to 2 g / kg per course). In the maintenance regimen for CIDP, IVIG can be administered according to different schemes: a) a total dose of 0.4 g / kg within 1 day, 1–2 times a week; b) 1 g / kg within 2–3 days every 3–4 weeks; c) 2 g / kg within 3–5 days every 4–6 weeks.

When the patient's condition stabilizes during IVIG therapy for 6–12 months, gradual dose reduction or an increase in the interval between injections is recommended. A combination of glucocorticosteroids (GCS) and IVIG is allowed, as well as administration of IVIG after completion of the course of plasmapheresis [6–8]. In our cohort of patients, the majority of individuals were diagnosed with CIDP, and in all but one case, positive dynamics was noted after 1–2 courses of immunotherapy. An exception was the case of atypical CIDP with an acute onset, etiologically associated with podocytopathy, characterized by an aggressive course and resistance to GCS, plasmapheresis, and IVIG. The features of the disease in the patient were the presence of a pronounced nephrotic syndrome caused by minimal change disease (non-proliferative glomerulonephritis with podocytopathy). Presumably, such clinical presentation of CIDP is typical when the target of an autoimmune attack is neurofascin, which is localized both in the peripheral nervous system and in the podocyte cytoskeleton [9].

Within 5 days after the infusion, there was no increase in neurological deficit, but then bulbar disorders and tetraplegia began to rapidly increase, leading to intubation and follow-up in the intensive care unit. In the future, a combination of cytostatic therapy (cyclosporine and rituximab) and corticosteroids (oral prednisolone) had a dramatic positive effect. The lack of an adequate response to immunoglobulin can probably be explained by the gradually progressive nephrotic syndrome.

The off-label use of IVIG may be effective in the treatment of paraproteinemic demyelinating polyneuropathy associated with MGUS with Ig A and Ig G [10, 11] and as second-line therapy in patients with inflammatory myopathies (dermatomyositis, polymyositis). The dose may vary from 0.4 g / kg to 2 g / kg, depending on the severity of clinical symptoms. Currently, there are no unambiguous data on the effectiveness of IVIG in inclusion body myositis and necrotizing autoimmune myositis [4, 12, 13].

With MMN, the effectiveness of IVIG has been proven as the only method of pathogen-specific therapy. The starting course dose should be at least 2 g / kg for 3–5 days. The total maintenance dose can be 1 g / kg for 2–3 days every 2–4 weeks or 2 g / kg for 3–5 days every 1–2 months, although in our work in one case it was possible to reduce it to 0.5 g / kg. As a rule, patients with MMN require long-term, continuous maintenance therapy with IVIG [14, 15].

Stiff person syndrome is a rare autoimmune neurological disease associated with the presence of antibodies to glutamate decarboxylase (GAD) and amphiphysin. Currently, there are no generally accepted schemes for its therapy, however, along with GCS, IVIG and plasmapheresis have proven efficient. IVIG is administered at a course dose of 2 g / kg, followed by repeated courses at a dose of 1 g / kg. The duration of treatment and the interval between injections are determined by the level of autoantibodies and the severity of clinical symptoms [16]. In our cohort, a patient diagnosed with stiff person syndrome received 7 courses of IVIG infusions, during which a steady decrease in the severity of clinical manifestations was observed.

AE is a group of rare immune-mediated inflammatory diseases of the central nervous system. Its clinical presentation is dominated by encephalopathy of non-infectious origin, associated with the presence of autoantibodies of different specificities (to proteins of presynaptic terminals – GAD or amphiphysin; to synaptic proteins in neurons – GABAa,b receptors, AMPA receptors, VGC channels, NMDA receptors, etc.). Along with GCS and plasmapheresis, IVIG is referred to as first-line therapy, with a recommended dose of 2 g / kg per course. Second-line drugs include rituximab and cyclophosphamide [17]. In our study, for the patient with ineffective previous treatment with rituximab, IVIG therapy was accompanied by stabilization of the disease course throughout the year.

IVIGs are not included in the standard treatment regimen for multiple sclerosis (MS) and NMOSD. However, in MS and NMOSD, they are used in certain clinical situations, namely in children, in pregnant women, sometimes in severe exacerbations or verified immunodeficiency [3, 18–20]. In NMOSD, therapy with IVIG preparations to prevent the development of relapses can be carried out in the presence of contraindications to immunosuppressants [20]. In our cohort, in one patient with NMOSD, an exacerbation

developed during pregnancy, and in the second patient, it developed due to lymphopenia, the genesis of which was initially unclear, and later SLE was diagnosed as a comorbid condition. IVIG treatment in both cases was accompanied by rapid suppression of disease intensity, in the second case – after ineffective anti-relapse therapy with GCS and rituximab.

Of course, our observational study has a number of significant limitations, such as a small sample and demographic heterogeneity of patients, analysis of a wide range of diseases, use of various IVIG drugs, and a lack of randomization and a comparison group. Nevertheless, the results of the study seem to be important evidence of the efficacy and safety of IVIG preparations in the treatment of a number of immune-mediated diseases of the nervous system. They are often used in situations when other approved treatments are not effective or contraindicated, or when there is no approved therapy for certain nosological entities.

CONCLUSION

The use of IVIGs for the treatment of rare severe neurological diseases is possible not only within the approved indications, but also in the off-label fashion, taking into account known data on the pathogenesis and after assessing the risk – benefit ratio for patients in accordance with the order of the Ministry of Health and Social Development of the Russian Federation No. 494 of 09.08.2005 "On the procedure for the use of medicines in patients for health reasons" and Federal Law No. 323-FZ of 21.11.2011. These drugs have a good efficacy / safety ratio and in some cases may be the only means of treating life-threatening conditions and preventing profound disability. Potential systemic infusion reactions (fever, myalgia, asthenia, nausea, anaphylaxis) can be prevented by premedication. Further studies of IVIG preparations are required to determine their optimal doses, frequency of administration, as well as the duration of therapy for various neurological diseases in order to scientifically substantiate the expansion of the list of diseases for their use in the standards of medical care for neurological patients.

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ЖУРНАЛ «БЮЛЛЕТЕНЬ СИБИРСКОЙ МЕДИЦИНЫ» ПРЕДСТАВЛЕН НА СЛЕДУЮЩИХ РЕСУРСАХ

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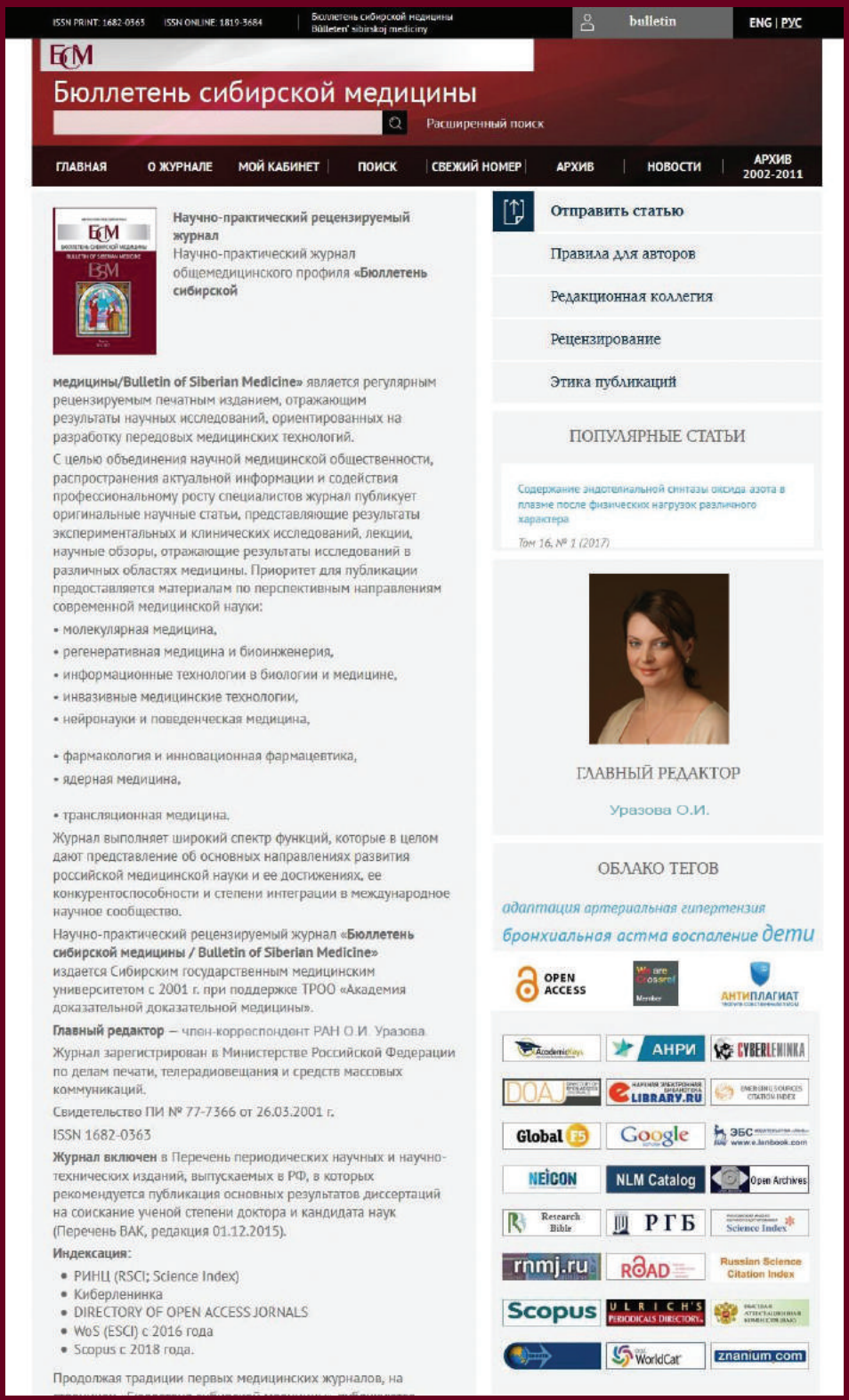

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