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Patterns of changes in immune and hormonal regulation in hand-arm vibration syndrome and sensorineural hearing loss

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ABSTRACT

The aim of the research was to identify changes in immune and hormonal regulation in patients with hand-arm vibration syndrome and sensorineural hearing loss to substantiate informative biomarkers.

Materials and methods. Men with occupational injury induced by exposure to vibration and noise were examined. The first group included 26 people diagnosed with stage 1 and 2 hand-arm vibration syndrome. The second group consisted of 38 patients diagnosed with sensorineural hearing loss. Serum levels of cortisol, dehydroepiandrosterone sulfate, prolactin, free triiodothyronine (T_3), free thyroxine (T_4), thyroid-stimulating hormone (TSH), and interleukins IL-1β, IL-8, IL-10 were determined by enzyme-linked immunosorbent assay.

Results. The results of the study revealed the peculiarities in the immune and hormonal regulation in hand-arm vibration syndrome and sensorineural hearing loss. More pronounced changes were observed in sensorineural hearing loss. A common pattern in patients with hand-arm vibration syndrome and sensorineural hearing loss was an increase in cortisol, prolactin and IL-8 and a decrease in free T_4 and IL-1β. Differences in the identified changes in the immune and hormonal status were characterized by increased TSH production in the first group, and increased free T_3 production and decreased IL-10 in the second group. In hand-arm vibration syndrome, high levels of cortisol were accompanied by a decrease in the IL-1β and IL-10 concentrations. In sensorineural hearing loss, an increase in the prolactin concentration was accompanied by increased production of IL-8.

Conclusions. The identified features of immune and hormonal relations may be induced by the intensity of cortisol and prolactin production under the effects of various physical factors. Persistent high levels of cortisol and prolactin in the examined patients are important pathogenetically significant factors in the development of the disease. New laboratory indicators (IL-4, prolactin, free T_3) for additional diagnosis of occupational sensorineural hearing loss were identified.

Key words: hand-arm vibration syndrome, sensorineural hearing loss, hormonal status, cytokines, diagnostic markers.

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

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Закономерности изменений иммуно-гормональной регуляции при вибрационной болезни и нейросенсорной тугоухости

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РЕЗЮМЕ

Цель исследования – выявить закономерности иммуно-гормональной регуляции при вибрационной болезни и нейросенсорной тугоухости для обоснования информативных биомаркеров.

Материалы и методы. Проведено обследование мужчин с профессиональной патологией, индуцированной воздействием вибрации и шума. В первую группу включены 26 пациентов с вибрационной болезнью I–II стадии, во вторую – 38 пациентов с профессиональной нейросенсорной тугоухостью. Методом иммуноферментного анализа в сыворотке крови определяли содержание кортизола, дегидроэпиандростерона сульфата, пролактина, свободного трийодтиронина, свободного тироксина, тиреотропного гормона (ТТГ); интерлейкина (IL) 1β, IL-8, IL-10.

Результаты. Результаты исследования позволили выявить особенности иммуно-гормональной регуляции при вибрационной болезни и нейросенсорной тугоухости. Общей закономерностью у пациентов с вибрационной болезнью и нейросенсорной тугоухостью являются возрастание кортизола, пролактина, IL-8 и снижение свободного (св.) T_4, IL-1β. Различия выявленных изменений в иммуно-гормональном статусе характеризовались для первых усилением продукции ТТГ, для вторых – возрастанием продукции св. T_3 и снижением IL-10. При вибрационной болезни высокие уровни кортизола сопровождались снижением концентрации IL-1β и IL-10, а при нейросенсорной тугоухости возрастание концентрации пролактина сопровождалось увеличением продукции IL-8.

Заключение. Выявленные особенности иммуно-гормональных взаимоотношений могут быть обусловлены интенсивностью выработки кортизола и пролактина при воздействии физических факторов различной природы. Сохраняющиеся высокие концентрации кортизола и пролактина у обследованных являются важными патогенетически значимыми факторами в развитии и течении заболеваний. Определены новые биомаркеры для дополнительной диагностики профессиональной нейросенсорной тугоухости (IL-4, пролактин, св. T_3).

Ключевые слова: вибрационная болезнь, нейросенсорная тугоухость, гормональный статус, цитокины, маркеры диагностики.

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INTRODUCTION

The problem of hand-arm vibration syndrome (HAVS) and occupational sensorineural hearing loss (SNHL) has retained its medical and social significance. These diseases have high prevalence and contribute to the disability of the working population. In this regard, they cause significant social and economic losses, since an occupational disease develops after 5–7 years of contact with the noise and vibration factor. In 3.6% of cases, it is diagnosed in people after 1–4 years of work in conditions of exposure to local vibration [1]. An important role in the pathogenesis of HAVS and SNHL is assigned to nervous, immune and endocrine regulation [2]. It is known that the effects of noise and vibration on the body of workers lead to the state of chronic stress. In this condition, hormones of the adrenal cortex inhibit the activity of cells of the immune system. In the long term, it results in a decrease in the body's resistance to infectious diseases, possible growth of various tumors, etc. [3]. Thus, studies allowing to identify violations of the immune and hormonal regulation remain relevant. In addition, the role of hormones in HAVS and SNHL is not well understood [4, 5]. The findings obtained in the studies may help take necessary measures to preserve health of workers and prevent work-related diseases.

The aim of the research was to identify changes in immune and hormonal regulation in patients with HAVS and SNHL and substantiate informative indicators.

MATERIALS AND METHODS

The study included 64 men with work-related diseases induced by exposure to noise and vibration. The first group included 26 people diagnosed with stage 1–2 HAVS. Their age was 48.9 ± 1.8 years and their occupational contact with vibration lasted 24.2 ± 1.9 years. The second group consisted of 38 patients diagnosed with SNHL. Their age was 54.1 ± 0.1 years and their occupational contact with noise lasted 31.1 ± 1.4 years. Patients were diagnosed by occupational therapists in accordance with the International Classification of Diseases, Tenth Revision (ICD-10). The comparison group consisted of 24 generally healthy men with matching age and employment time, who did not have occupational contact with vibration and noise.

Serum levels of cortisol, dehydroepiandrosteron sulfate (DHEA-S), prolactin, free triiodothyronine (free T_3), free thyroxine (free T_4), and thyroid-stimulating hormone (TSH) were determined by enzyme-linked immunosorbent assay (Alkorbio, St. Petersburg, Russia). The same method was used to measure the levels of cytokines IL-1β, IL-8 and IL-10 (Vector-Best, Novosibirsk, Russia).

The results were analyzed using STATISTICA 6.0 software package (StatSoft, the USA). The age and employment time of the patients were presented as a mean *(M)* and a mean error (*m*). The results of the studies were presented in form of a median (*Me*) and upper (75%) and lower (25%) quartiles (*Q25–Q75*). The groups were compared using the Friedman test. Significance of differences was estimated using the nonparametric Wilcoxon-Mann-Whitney test with the Bonferroni correction. Correlation analysis was performed using the Spearman's rank correlation coefficient. The differences were statistically significant at $p < 0.05$. Discriminant analysis was performed according to the manual for physicians "Discriminant Analysis in Biomedical Research" (using STATISTICA 6.1 [8] and the Discriminant Analysis module). The informativity of the analyzed indices was assessed in evaluation steps using the cutoff $F > 3.0$. The Mahalanobis Distance Classification D2 was used as the classification criterion.

RESULTS

We have previously shown that workers with employment experience exposed to vibration have activation of immune cells with the release of IL-1β and TNFα, which contribute to the production of glucocorticoids [3]. Glucocorticoids can inhibit the immune system. Steroid hormones of the adrenal cortex are regulators of vital processes, such as coordinated growth, differentiation, reproduction, adaptation, and behavior [6]. Based on this knowledge, we conducted a comparative evaluation of the levels of glucocorticoid and thyroid hormones in patients with HAVS and SNHL (Table 1).

The results of the study showed an excess of cortisol in serum of patients with HAVS by 1.7 times ($p = 0.043$) and in patients with SNHL by 2.8 times ($p = 0.0003$), in contrast to the comparison group. A more significant increase in cortisol levels was observed in SNHL patients compared to HAVS

patients (p = 0.001). In patients with HAVS, a decrease in TSH level was registered, as opposed to SNHL patients (p = 0.00003) and the comparison group (p = 0.002).

Table 1

Hormone levels in patients with hand-arm vibration syndrome (HAVS) and sensorineural hearing loss (SNHL), *Me* (Q_{25}–Q_{75})

Parameter	Unit	Patients with HAVS, n = 26	Patients with SNHL, n = 38	Comparison group, n = 24
Cortisol	nmol/l	648.7 (278.9–803.6)♦	1085.2 (795.1–1296.7)♦*	378.7 (211.9–532.4)
DHEA-S	nmol/l	1.4 (1.2–1.9)	1.5 (1.1–1.9)	1.7 (1.3–1.9)
Prolactin	mIU/L	117.9 (19.8–184.9)♦	254.9 (180.1–297.4)♦*	55.8 (0.001–109.9)
Free T_3	nmol/l	4.6 (4.3–5.1)	5.4 (3.6–6.4)♦	3.8 (3.2–4.8)
Free T_4	nmol/l	11.7 (9.5–12.8)♦	13.8 (12.2–14.7)♦*	15.5 (14.1–16.7)
TSH	mIU/L	0.7 (0.5–1.3)♦	1.7 (1.2–2.3)*	1.3 (1.0–1.6)

♦ differences as opposed to the comparison group; *differences between the groups of patients with HAVS and SNHL are statistically significant at $p < 0.016$.

An increase in the concentration of free T_3 was found only in patients with SNHL (p = 0.016). The free T_4 concentration decreased both in patients with HAVS (p = 0.00001) and in patients with SNHL (p = 0.005) relative to the comparison group. An increase in prolactin concentration by 2.1 times (p = 0.04) was detected in patients with HAVS, and by 4.5 times (p = 0.001) in patients with SNHL relative to the comparison group. A significant increase (p = 0.002) in this parameter was observed in SNHL patients in contrast to HAVS patients. Prolactin is known to stimulate the production of a number of cytokines [7]. A significant prolactin increase (p = 0.002) was observed in SNHL patients in contrast to HAVS patients.

The detected hormonal changes in the examined patients were accompanied by cytokine balance disorder (Table 2).

Table 2

Serum cytokine levels in patients with hand-arm vibration syndrome and sensorineural hearing loss, *Me* (Q_{25}–Q_{75})

Parameter, pg / ml	HAVS, n = 26	SNHL, n = 38	Comparison group, n = 24
IL-1β	0.01 (0.01–0.1)♦	0.01 (0.01–2.7)	0.9 (0.5–1.8)
IL-8	6.8 (5.7–8.3)♦	8.7 (4.1–20.5)♦	2.7 (1.8–3.1)
IL-4	0.01 (0.01–0.02)	0.01 (0.01–0.01)	0.01 (0.01–8.8)
IL-10	1.1 (0.01–1.66)	0.01 (0.01–0.01)♦	2.1 (0.01–13.4)

♦ differences as opposed to the comparison group are statistically significant at $p < 0.016$.

A significant decrease in the concentration of IL-1β (0.01 (0.01–0.1) pg/ml, p = 0.013) in patients with HAVS and a pronounced trend towards a significant decrease in patients with SNHL (0.01 (0.01–2.7) pg/ml, p = 0.056) were revealed. High IL-8 values (p = 0.002) were determined in patients with HAVS and SNHL, as opposed to the comparison group (p = 0.0047 and p = 0.0016, respectively). A decrease in IL-10 was registered in patients with SNHL only (0.01 (0.01–0.01) pg/ml), in contrast to the comparison group [2.1 (0.01–13.4) pg/ml; p = 0.015].

At the next stage, the correlation between changes in the hormonal status and cytokine levels was revealed. Following the correlation analysis, it was revealed that patients with HAVS had a negative correlation between cortisol level and IL-1β level (r = –0.49; p = 0.018) and a positive correlation between cortisol level and IL-10 level (r = 0.49; p = 0.018). Patients with SNHL showed a positive correlation between prolactin level and IL-8 production (r = 0.44; p = 0.009).

Informative indicators for SNHL diagnosis were found with the help of discriminant analysis; 23 immunological indicators were processed from the group of patients with SNHL and the comparison group.

As a result of the calculations, the most significant criteria were IL-4 (p = 0.0045), prolactin (p = 0.0098), and free T_3 (p = 0.0227). The accuracy of SNHL diagnosis equaled 98%.

DISCUSSION

Comparative estimation of glucocorticoid and thyroid hormone levels in patients with HAVS and SNHL revealed both common patterns and distinctive features of their concentration. Both HAVS and SNHL patients were found to have exceeded cortisol levels. In patients with SNHL, cortisol production was 2 times higher than in patients with HAVS. The expected differences in DHEA-S level were not revealed in patients with HAVS and SNHL. It is known that long-term cortisol excess may be accompanied by impaired sensitivity of adrenal cortex cells to adrenocorticotropic hormone and, consequently, by a decrease in DHEA production. Due to its biological effects, DHEA is considered as an inhibitor to the effects of cortisol on various body systems (especially the immune system and the brain) [8–10].

Individual authors note that there is a decrease in the production of DHEA-S with not changing or slightly increasing level of cortisol in some diseases accompanied by central nervous system (CNS) disorders. It results in a decrease in the DHEA/cortisol ratio [11]. A twofold increase in prolactin level is seen in patients with SNHL compared to patients with HAVS. Prolactin affects the immune system under stress [12], stimulating cytokine production, T-cell proliferation, NK cells, neutrophils, and dendritic cells [13]. A decrease in the average free T_4 and IL-1β levels and increased IL-8 are a common pattern for both HAVS and SNHL. A specific feature of the detected changes in the patients with SNHL is the increase in free T_3 production and the decrease in IL-10 level. Patients with HAVS are characterized by increased TSH production. As it is known, free T_3 and free T_4 are involved in the regulation of TSH emissions [14]. Free T_3 is the main TSH suppressor, as its high concentrations block TSH production, while low levels increase it [15].

According to the work of R.G. Fedina. et al., there is a significant increase in cortisol and a decrease in free T_3 and free T_4 even in apparently healthy workers exposed to vibration hazards [16]. V.S. Rukavishnikov and A.V. Lizarev demonstrated that only insignificant changes of the mentioned hormones are noted in patients with HAVS along with an increase in the length of service [17]. The results of the examination of workers in the machine-building industry exposed to increased levels of vibration and noise may be found in literature. The workers showed an increase in adrenocorticotropic and luteotropic activity of the adenohypophysis and a decrease in androgen production [18].

These results confirm that prolonged exposure to high doses of cortisol and prolactin contributes to the development of various disorders in the immune system regulation [19]. Thus, in patients with HAVS, high cortisol level was accompanied by a decrease in IL-1β and an increase in IL-10 concentrations. In patients with SNHL, prolactin production had a positive correlation with IL-8 production. IL-1 is a multifunctional cytokine. It easily penetrates the brain through the blood-brain barrier and causes secretion of corticotropin-releasing factor in the hypothalamus. This factor affects the functional activity of the pituitary and adrenal glands. In response to that, the pituitary gland stimulates adrenocorticotropic hormone secretion. The adrenal cortex stimulates glucocorticoid hormones. There is experimental evidence that cytokines have both immune and neurotropic effects. They are produced in the CNS and can have a direct effect on the nervous system [20, 21].

The established differences in immune and hormonal relationships may be induced by the intensity of cortisol and prolactin production in HAVS and SNHL. The findings prove that high concentrations of cortisol and prolactin in patients with HAVS and SNHL are important pathogenetically significant factors in the development of the diseases. Thus, new informative laboratory indicators (IL-4, prolactin, free T_3) can be used to identify changes in immune and hormonal regulation in patients with SNHL.

CONCLUSION

The patterns of immune and hormonal regulation in occupational HAVS and SNHL were revealed. The common pattern of changes in hormonal and cytokine profiles in patients with HAVS and SNHL was the following: increased levels of cortisol, prolactin, IL-8 and decreased levels of free Th_4 and IL-1β. A distinctive feature of occupational SNHL was the increase in free T_3 production and the decrease in anti-inflammatory IL-10 level. In patients with HAVS, the increase in TSH production was identified. It was found that patients

with HAVS had a negative correlation between the cortisol level and the concentration of pro-inflammatory IL-1β and a positive correlation between cortisol and anti-inflammatory IL-10. Patients with SNHL had a positive correlation between the prolactin concentration and IL-8 production. The identified features of immune and hormonal relationships may be determined by the intensity of cortisol and prolactin production. Persistent high levels of cortisol and prolactin are important pathogenetically significant factors in the development of HAVS and SNHL. The obtained new informative laboratory indicators (IL-4, prolactin, free T_3) will allow to expand the evidence base for the diagnosis of SNHL.

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Somatic pathology in residents of Khanty-Mansi Autonomous Okrug – Yugra

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ABSTRACT

Aim. To study the 8-year dynamics of somatic pathology in residents of the Khanty-Mansi Autonomous Okrug – Yugra.

Materials and methods. The article analyzes the migration of the population of the Far North and the dynamics of the incidence of chronic non-infectious pathology among residents of territories equated to the Far North – the Khanty-Mansi Autonomous Okrug – Yugra, based on literature data and officially registered statistics for clinical and statistical groups for the period 2010–2017.

Results. The analysis revealed the leading groups of somatic pathology in the Khanty-Mansi Autonomous Okrug – Yugra. The indicators of population dynamics of the territories of the Far North of Russia were estimated.

Conclusions. The study identified patterns in different flows of the Russian population in and from the North, the incidence rate (defined by the leading group of diseases) and its dynamics, characteristic of the territories equated to the Far North. The obtained data make it possible to identify priority research areas aimed at analyzing the frequency of diseases of internal organs in the territories equated to the Far North, the features of their course and outcomes as well as to develop effective programs of primary and secondary prevention of these diseases.

Key words: Far North, morbidity, somatic pathology, metabolic syndrome, steatohepatitis, respiratory diseases, diseases of the digestive system, hypertension, coronary heart disease, opisthorchiasis.

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Соматическая патология у жителей Ханты-Мансийского автономного округа – Югры

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РЕЗЮМЕ

Цель. Изучение 8-летней динамики соматической патологии у жителей Ханты-Мансийского автономного округа – Югры (ХМАО – Югры).

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Материалы и методы. В статье проведен анализ миграции населения Крайнего Севера, изучена динамика заболеваемости хронической неинфекционной патологией у жителей территории, приравненной к Крайнему Северу, – ХМАО – Югры. Использованы данные литературы и официально регистрируемой статистики по клинико-статистическим группам за период 2010–2017 гг.

Результаты. Выявлены лидирующие группы соматической патологии в Ханты-Мансийском автономном округе – Югре. Оценены показатели динамики населения территорий Крайнего Севера России.

Заключение. Полученные данные позволяют определить приоритеты для научных исследований, направленных на анализ частоты болезней внутренних органов на территориях, приравненных к Крайнему Северу, особенностей их течения и исходов, а также разработку эффективных программ первичной и вторичной профилактики этих заболеваний.

Ключевые слова: Крайний Север, заболеваемость, соматическая патология, метаболический синдром, стеатогепатит, болезни органов дыхания, болезни пищеварительной системы, артериальная гипертензия, ишемическая болезнь сердца, описторхоз.

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

Health problems of the global population are caused by many factors, primarily genetic and environmental ones. If we are evaluating human health, it is important to understand the degree of influence of climatic and geographical factors (primarily, cold and increased geomagnetic activity); we also need to keep in mind the technogenic factors. Due to migration of the population to economically developed regions of the world and to the Far North and equivalent territories, it is necessary to control the state of health of the indigenous population and people coming from other geographical locations [1].

The northern territories occupy 20% of the globe, and in Russia, 7.4% of the population live in 11 northern regions (beyond the 60th parallel). [2] The growing interest in the Northern territories is primarily explained by abundance of mineral resources such as gas, diamonds, oil, etc., as well as by the rapidly developing mining industry.

Migration in most regions of the Far North and their equivalent territories is ambiguous. On the one hand, it happens due to complete or partial urbanization of the small indigenous minorities of the North, and on the other hand, due to relocation of the working-age population of the country from favorable climatic conditions of life to unfavorable and difficult ones to work in national and international corporations [2–4]. Migration processes are complex for both the first and the second categories of the population. In this regard, the processes of maladaptation of the human body are developing, and these processes are characterized by tension and restructuring of homeostasis [4, 5]. The problem of human life in the northern latitudes is determined by survivability in extreme situations and the necessity to maintain good health in complex interactions with new technogenic, environmental, social, and psychological factors [3, 6, 7]. Change in the nature of nutrition plays a significant role, especially for the indigenous minorities of the North, who are accustomed to their culture and stereotypes that have developed over the centuries [1, 4, 7].

Taking into account the fact that the risk of developing internal organ diseases increases significantly in the North [7–10], it is extremely important to develop measures aimed at improving the quality of life and increasing the life expectancy of a person living in atypical and extreme conditions within the P5 paradigm of medicine [11, 12].

Clinical epidemiology, as a tool of evidence-based medicine, allows to objectively assess the situation with non-infectious pathology that dominates the structure of mortality and justify the measures for targeted correction of negative effects in high-risk groups [6, 12, 13].

The aim of this research was to carry out a comparative study of the parameters and structure of the most common somatic pathology in the residents of the Khanty-Mansi Autonomous Okrug – Yugra (KhMAO – Yugra).

MATERIALS AND METHODS

Demographic parameters and incidence rates of the residents of KhMAO – Yugra have been analyzed

using a continuous sampling method during an 8-year period, from 2010 to 2017. We took the data on the size and migration of the Russian population [14] from official statistics and collections prepared by the Department of Monitoring, Analysis, and Strategic Development in Healthcare of the Ministry of Health of the Russian Federation. The data were also provided by the Federal Research Institute for Health Organization and Informatics of the Ministry of Health of the Russian Federation. The clinical and statistical groups were formed on the basis of official reporting statistics forms No. 12 "Information about the number of diseases registered in patients living in the area of examination of the medical institution" and No. 14 "Information about the hospital activities".

The dynamics of the most important morbidity parameters was analyzed, including long-term annual average values for somatic systems of organs. The study was conducted as part of the research topic of the Department of Internal Medicine of Surgut State University, approved on 24.06.2019. The initiative theme of the R&D No. AAAA-A19-119062490051-6 "Predictors of the genesis, course and outcome of chronic and comorbid diseases" was registered in the Unified State Information System for Recording the Results of Research, Developments and Technological Works.

RESULTS AND DISCUSSION

Analysis of the dynamics of migration flows from central Russia to the northern regions and migration flows from them allows to assess the demographic situation in the country (Table 1). As of 01.01.2018, 146, 880, 432 residents lived in the Russian Federation.

9, 920, 891 people lived in 26 regions of the Far North and the territories equated to them, which is 6.75% of the whole population of the country or every 14th-15th resident (Table 1).

Table 1

The number of inhabitants and migration trends in the territories of the Far North and areas equated to them in 2018*

№	Region	Population as of 01.01.2018	Total growth	Natural growth	Migration growth
Regions of the Far North and areas equated to them, in total		9, 920, 891	–37, 895	12, 975	–50, 870
1	Altai Republic	30, 762	213	446	–233
2	Republic of Buryatia	94, 897	–1, 283	120	–1, 403
3	Republic of Karelia	622, 484	–4, 428	–3, 108	–1, 320
4	Komi Republic	840, 873	–10, 638	–1, 362	–9, 276
5	Sakha Republic	964, 330	2, 679	5, 619	–2, 940
6	Tyva Republic	321, 722	2, 701	3, 681	–980
7	Zabaikalsky Krai	21, 041	–440	–13	–427
8	Kamchatka Krai	315, 557	–834	–132	–702
9	Krasnoyarsk Krai	439, 276	–133	1, 201	–1, 334
10	Perm Krai	28, 387	–393	–77	–316
11	Primorsky Krai	99, 173	–1, 676	–687	–989
12	Khabarovsk Krai	515, 285	–5, 610	–2, 064	–3, 546
13	Amur Oblast	94, 690	–1, 380	–297	–1, 083
14	Arkhangelsk Oblast including the Nenets Autonomous Okrug	1, 155, 028	–10, 909	–3, 816	–7, 093
15	Nenets Autonomous Okrug	43, 997	–168	224	–392
16	Arkhangelsk Oblast without the Autonomous Okrug	1, 111, 031	–10, 741	–4, 040	–6, 701
17	Irkutsk Oblast	534, 792	–6, 712	–1, 772	–4, 940
18	Magadan Oblast	144, 091	–2, 857	–194	–2, 663
19	Murmansk Oblast	753, 557	–5, 501	–1, 099	–4, 402
20	Sakhalin Oblast	490, 181	–543	–219	–324
21	Tomsk Oblast	192, 620	–2,120	–190	–1, 930
22	Tyumen Oblast including the Autonomous Okrug	2, 212, 797	11, 654	16, 860	–5, 206
23	Khanty-Mansi Autonomous Okrug – Yugra	1, 655, 074	8, 721	12, 145	–3, 424

Table 1 (continued)

24	Yamalo-Nenets Autonomous Okrug	538, 547	2, 932	4, 667	–1, 735
25	Tyumen Oblast without the Autonomous Okrug	19, 176	1	48	–47
26	Chukotka Autonomous Okrug	49, 348	315	78	237

*http://www.rosstat.gov.ru/wps/wcm/connect/rosstat_main/rosstat/ru/statistics/publications/catal og/doc_1140096034906

Official data from Rosstat show that in 25 out of 26 regions mainly negative migration statistics was revealed, and only 12 regions had positive dynamics with natural population growth. Having analyzed the migration processes in 11 regions of the Far North, it can be noted that 10 of them have outflow of the population. In 2018 alone, 271, 384 people arrived in the Far North of Russia and 305, 382 people left it, so the migration loss of the population in this year was 33, 998 people in 10 out of 11 regions (table 2).

Table 2

Dynamics of migration of the population in the Far North regions in 2018*

№	Region	Number of immigrants (*n* =)	Number of emigrants (*n* =)	Dynamics %
1	Republic of Karelia	22, 161	23, 331	–1.05
2	Komi Republic	31, 695	40, 484	–1.28
3	Nenets Autonomous Okrug	2, 309	2, 566	–1.11
4	Murmansk Oblast	35, 460	39, 866	–1.12
5	Khanty-Mansi Autonomous Okrug -Yugra	66, 390	72, 549	–1.09
6	Yamalo-Nenets Autonomous Okrug	30, 549	34, 211	–1.12
7	Tyva Republic	12, 307	13, 406	–1.09
8	Sakha Republic	39, 226	44, 404	–1.13
9	Magadan Oblast	6, 629	8, 707	–1.31
10	Sakhalin Oblast	19, 383	20, 769	–1.07
11	Chukotka Autonomous Okrug	5, 275	5, 089	+1.04
	Total	271, 384 people	305, 382 people	–33, 998 people

*http://www.rosstat.gov.ru/wps/wcm/connect/rosstat_main/rosstat/ru/statistics/publicatio ns/catalog/doc_1140096034906

In 2018, the Sakhalin Oblast, KhMAO – Yugra and the Republic of Tyva were the most attractive of the 11 Northern territories of Russia, with 1.07% – 1.09% of the population leaving them (Table 2). The largest number of migrant's returns was registered in the Republic of Komi – 8, 789 people, or 1.28% , and the lowest – in the Chukotka Autonomous Okrug, from which only 186 people left (+1.04%).

As of 01.01.2018 and 01.01.2019, KhMAO – Yugra had 1, 655, 074 and 1, 663, 795 residents, respectively, so the total population growth was 1.5%. As of 01.01.2019, 1.14% of the total population of Russia lived in KhMAO – Yugra. The population of this region is primarily young, aged 34–39 years.

Due to the common climate, geographical and environmental characteristics of all northern territories, the results of the study on epidemiological indicators in one of them can be extrapolated to all northern territories of the Russian Federation and the globe. At the same time, human life expectancy in the North is very important, since people migrating to other climatic territories are initially burdened with chronic somatic diseases [3].

In KhMAO – Yugra, the mortality rate is generally lower than in other regions of the country, but among working age population it reaches 73.5% of the total number of deaths, which necessitates a detailed study of the causes of this situation and development of measures for practical healthcare [3, 9, 13].

Studying the officially registered morbidity rates in Russia and in the territories of the Tyumen Oblast and KhMAO – Yugra, we obtained the following data. According to the long-term annual average indicators of the general morbidity of the population, KhMAO – Yugra parameters did not differ from the all-Russian ones (Fig.1). However, in the North, there was a progressive increase in the morbidity of the population despite their

young age (Fig. 2). The total morbidity of the population in KhMAO – Yugra is 10.7% – 10.5% higher than the morbidity rate of the population in the Ural Federal District (UrFD) and the Tyumen Oblast, excluding KhMAO – Yugra (Fig. 1).

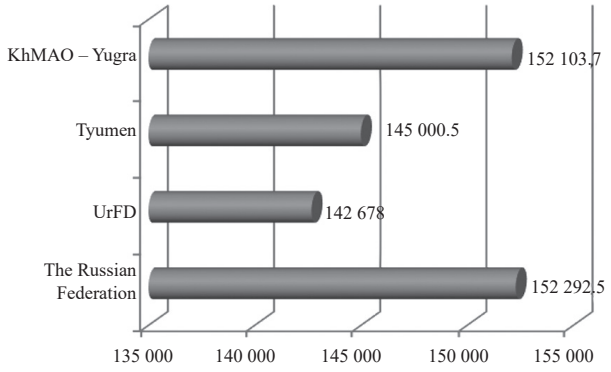

Fig. 1. Weighted average indicators of general morbidity of the population for the period 2010–2018 (per 100, 000 population)

Regional indicators of morbidity were compared with indicators throughout Russia, in the UrFD and the Tyumen Oblast, without taking into account the population of KhMAO – Yugra. During the analysis of the health status of the population of KhMAO – Yugra, we identified the following facts (Fig. 1 – 2).

Over the course of eight years, the long-time annual average rates of morbidity per 100, 000 population in the compared territories varied from 142, 678 to 152, 292.5 [5]. The overall morbidity of the young population in KhMAO – Yugra did not differ much from the national average value. The morbidity was 9, 425.7 higher than in the UrFD and 7, 103.2 higher than in the Tyumen Oblast (Fig. 1). In addition to the highest morbidity rates in KhMAO – Yugra, it had progressive growth rates, compared with the general morbidity in the Russian Federation (Fig. 1).

Amongst somatic pathologies, the leading ones are diseases of the respiratory, cardiovascular, digestive and endocrine systems, which is confirmed by the literature [2].

Despite the young age of KhMAO – Yugra population, respiratory diseases in the region have prevailed over similar indicators throughout Russia since 2010, apart from indicators in the Tyumen Oblast and the Ural Federal District with an annual progressive increase starting in 2013 (Fig. 2).

In KhMAO – Yugra, the incidence of cardiovascular diseases is the lowest in comparison with other regions and Russia on the whole. The fluctuations in new cases of cardiovascular diseases in different years was registered (Fig. 3).

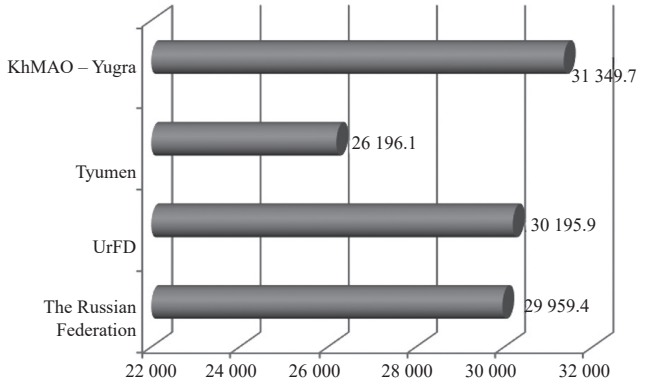

Fig. 2. Weighted average morbidity rates for the period 2010–2018 (pathology of the respiratory system per 100, 000 population)

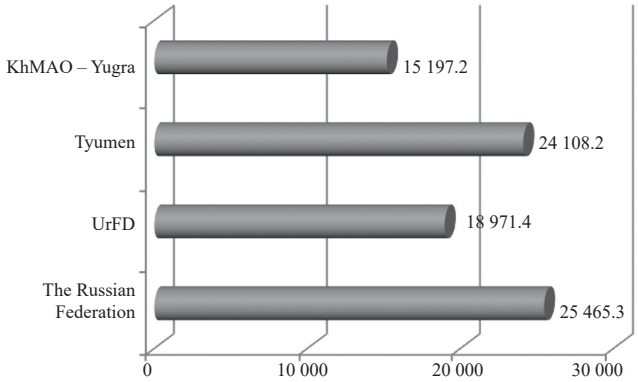

Fig. 3. Weighted average morbidity rates for the period 2010–2018 (pathology of the cardiovascular system per 100, 000 population)

The pathology of the cardiovascular system is predominantly represented by arterial hypertension and coronary heart disease. Non-coronarogenic myocardial diseases, pericarditis and acute rheumatic fever are less common, but there is high prevalence of atherosclerosis of various localizations and infectious endocarditis officially registered in other groups of pathologies.

The pathology of the digestive system is much more common in KhMAO – Yugra than in the Ural Federal District and the Tyumen Oblast (Fig. 4) and also has progressive growth rates (Fig. 4). This may be explained by the widespread prevalence of opisthorchiasis in the Ob – Irtysh basin [14]. Moreover, the increase in the incidence of steatohepatitis, cardiovascular pathology in view of the cardiovascular disease continuum and hypertension, which form the paradigm of the metabolic syndrome [15], is confirmed by the dynamics of the incidence of type 2 diabetes and obesity in KhMAO – Yugra (Fig. 5)

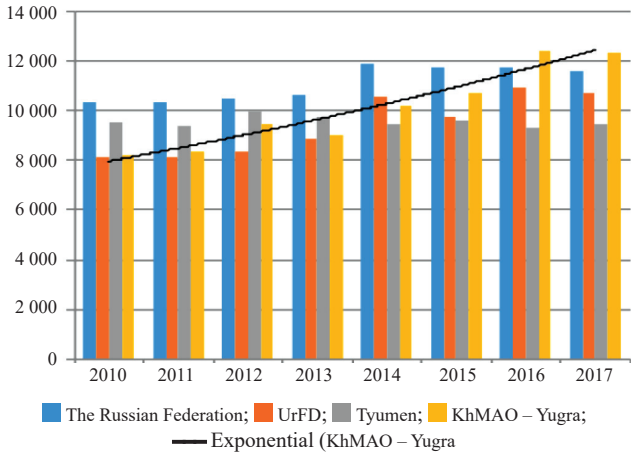

Fig. 4. 8-year dynamics of morbidity rates in KhMAO – Yugra and other regions of Russia (pathology of the digestive system per 100, 000 population)

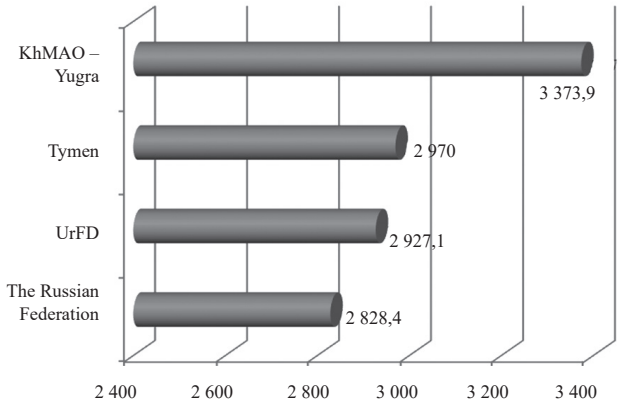

Fig. 5. Weighted average incidence of type 2 diabetes for the period 2010–2018 (per 100, 000 population)

The incidence of type 2 diabetes in the Ural Federal District and the Tyumen Oblast is higher than in Russia on the whole, which may serve as a marker of the metabolic syndrome of residents of the Cisurals, the North and territories equated to it.

Obesity is equally common in the Ural Federal District, in the Far North and in the Tyumen Oblast, but the number of obese people in these territories is still higher than in the rest of Russia (Fig. 6). This indicator characterizes the progressive growth of the disease among residents of the Tyumen Oblast and KhMAO – Yugra, despite young working age population and large migration flows, which can be seen from the data on trend lines.

Thus, in the Khanty-Mansi Autonomous Okrug – Ugra with minimal outflow of the population only in 2018, young age of residents and morbidity parameters comparable with the all-Russian ones, an increase in metabolic disorders is recorded, primarily of obesity and type 2 diabetes.

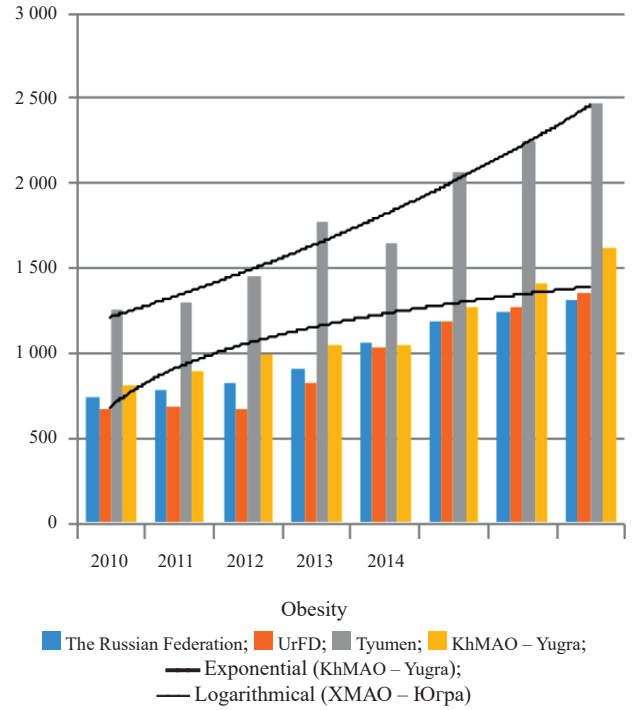

Fig. 6. 8-year dynamics of morbidity rate of obesity in the population of KhMAO –Yugra (per 100, 000 population)

CONCLUSION

We have analyzed the morbidity of the main classes of internal organ pathologies in people living in the economically developed territories of Russia, which are equated to the Far North. KhMAO – Yugra attracts the largest number of migrants from all over the country, while the smallest number of people leaves the district. The region has the highest and progressing morbidity of respiratory diseases and pathologies forming the metabolic syndrome – diabetes and obesity. In KhMAO – Yugra, the largest number of patients suffer from type 2 diabetes, obesity and digestive diseases, in particular, steatohepatitis.

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Authors contribution

Verizhnikova L.N. – design and interpretation of data. Aryamkina O.L. – verification of intellectual content, final approval of the article for publication. Terentyeva N.N. – design and interpretation of data.

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Structural and functional indicators of erythrocyte membranes in gastric cancer patients with different histotypes of the tumor and stages of the malignant process

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ABSTRACT

The aim was to study the structural and functional parameters of erythrocyte membranes in the blood of patients with gastric cancer (GC) – adenocarcinoma, depending on its grade, signet ring cell carcinoma (SRCC), and combined gastric lesions (CGL).

Materials and methods. The membrane fluidity in the area of the lipid bilayer and protein-lipid contacts, the polarity of the lipid bilayer and the immersion of proteins in the lipid matrix of the membrane in red blood cells were evaluated by fluorimetry using the hydrophobic pyrene-based probe. The study included 86 patients with GC divided into six groups: well- and moderately-differentiated adenocarcinoma (G1-2); poorly-differentiated adenocarcinoma (G3); SRCC; CGL and two groups of patients with a component of undifferentiated cancer: G4 + SRCC and G4 + G2-3. The results of the study were also analyzed in patients with serosal invasion and the spread to adjacent structures (T4 according to the TNM classification of malignant tumors) and in patients with stage IV disease.

Results. In all groups of GC patients, an increase in the membrane fluidity was observed. It was more pronounced in the zone of protein-lipid contacts, but it was also observed in the lipid bilayer. The membrane fluidity increased together with the grade of adenocarcinoma and was maximal when there were undifferentiated cells in stomach tumors, reaching 93.8% in the zone of protein-lipid contacts and 54.1% in the lipid bilayer, compared with healthy people (20 donors). An increase in the polarity of the lipid phase was also observed; it was most pronounced (by 7–8%, $p = 0.001$ 0.003) in adenocarcinoma patients with undifferentiated cells and with stage IV disease. A change in the immersion of proteins in the lipid matrix of erythrocytes was less characteristic of GC, compared with other cancers (breast, lung tumors, gynecological oncopathology, etc.).

Conclusions. Changes in the structural and functional properties of erythrocyte membranes reflect the state of the disease in patients with gastric cancer and may be important for predicting the course of the disease and the success of treatment.

Key words: gastric cancer, various tumor histotypes and cancer stages, erythrocyte membrane, fluidity, polarity, protein immersion, pyrene-based fluorescent probe.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study protocol was approved by the Ethics Council of Rostov Research Institute of Oncology (Protocol No. 11/1, dated 03.11.2016).

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Структурно-функциональные показатели мембран эритроцитов крови больных раком желудка с разным гистотипом опухоли и стадией злокачественного процесса

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РЕЗЮМЕ

Цель. Изучить структурно-функциональные показатели мембран эритроцитов в крови больных раком желудка (РЖ) при аденокарциноме, в зависимости от степени ее дифференцировки, при перстневидноклеточном раке (ПКР) и сочетанном поражении желудка (СПЖ).

Материалы и методы. Оценивали текучесть мембран в области липидного бислоя и белок-липидных контактов, полярность липидного бислоя и погруженность белков в липидный матрикс мембраны в эритроцитах крови с использованием гидрофобного зонда пирена флуориметрическим методом. В исследование было включено 86 больных РЖ, в зависимости от гистотипа разделенных на шесть групп: G1-2, G3, ПКР, СПЖ, G4 + ПКР и G4 + G2-3. Отдельно были проанализированы результаты исследования у больных с прорастанием опухоли в серозную оболочку и распространением на соседние структуры (T4 по системе классификации TNM) и у больных, находившихся в IV стадии.

Результаты. Во всех группах больных РЖ установлено увеличение текучести мембран, более выраженное в зоне белок-липидных контактов, но наблюдавшееся и в липидном бислое. При этом текучесть возрастала по мере снижения степени дифференцировки аденокарциномы и была максимальной при наличии в опухоли желудка недифференцированных клеток: выше, чем в группе здоровых, на 93,8% в зоне белок-липидных контактов и на 54,1% в липидном бислое. Наблюдалось также повышение полярности липидной фазы, наиболее выраженное (на 7–8%, $p = 0{,}002–0{,}003$) у больных аденокарциномой с наличием недифференцированных клеток и при IV стадии процесса. Изменение погруженности белков в липидный матрикс эритроцитов было менее характерно для РЖ по сравнению с другими раками (молочной железы, легкого, онкогинекологическая патология).

Заключение. Изменение структурно-функциональных свойств мембран эритроцитов отражает состояние процесса у больных раком желудка и может иметь значение для прогнозирования течения заболевания и успешности лечения.

Ключевые слова: рак желудка, различные гистотипы опухоли и стадии процесса, мембраны эритроцитов, текучесть, полярность, погруженность белков, флуоресцентный зонд пирен.

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

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Протокол исследования одобрен советом по этике ФГБУ «Ростовский научно-исследовательский онкологический институт» (протокол № 11/1 от 03.11.2016).

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INTRODUCTION

According to the data of modern lipidomics, membrane lipids play an important role in the implementation of many cell functions and are involved in the development of a number of pathologies, including cancer [1]. The revealed compositional features of various membranes (outer and inner layers of the outer membrane, organelle membrane) of tumor cells in different types and stages of cancer open up opportunities for new strategies in treatment and prevention of cancer [2]. Among the nonspecific disorders of homeostasis that develop following the effect of a tumor on the body are changes in the structural and functional properties of the membranes of peripheral blood cells, primarily erythrocytes [3, 4]. In diseases of various origin, erythrocytes undergo both specific and nonspecific structural, functional, and metabolic rearrangements; therefore, when studying various physiological processes and pathological conditions, they are considered as a universal cellular model reflecting changes in the body [5]. It is assumed that the study of the functional state of erythrocyte membranes in cancer patients is very important for early diagnosis of the disease and monitoring the success of treatment [6]. The authors came to this conclusion after studying the dielectric constant determined by structural changes in the membranes, in erythrocytes of 28 healthy donors and 62 patients with breast and lung cancer.

Studying membranes with fluorescent probes provides important information about the structural state of membranes. The fluorescence parameters of the probe introduced into the membrane depend on the physicochemical properties of its direct microenvironment in the membranes, such as fluidity, polarity of the medium, proximity of charged groups, the presence of various acceptor molecules, electron excitation energy, and diffusion of fluorescence quenchers [7, 8]. The use of a hydrophobic pyrene-based probe allows to evaluate the fluidity of blood cell membranes in the zones of protein-lipid contacts and the lipid bilayer, the polarity and immersion of proteins in the lipid matrix. Fluidity is a complex indicator that reflects both the structure and the basic properties of the lipid component of membranes and zones of protein-lipid interactions; it plays a key role in the regulation of all processes occurring in cell membranes. Fluidity characterizes not only the state of membranes, but also their ability to react to changes in the body in response to development of the malignant process and effects of chemotherapy [1, 9–11]. It has been proven that induction of apoptosis by many antitumor agents is associated with a change in the fluidity of tumor cell membranes under their influence [12]. The polarity of the lipid bilayer reflects the state of the hydrophobic phase of the membrane and the level of hydrophilic clusters formed by hydroperoxides in it. Assessment of the immersion of proteins in the lipid matrix allows to judge about the association of proteins with membranes, as well as about the oligomerization of membrane proteins which may result from their oxidative modification [8].

The aim of this study was to assess the structural and functional parameters of erythrocyte membranes in the blood of patients with gastric cancer – adenocarcinoma, depending on its grade, signet ring cell carcinoma, and combined gastric lesions.

MATERIALS AND METHODS

The study included 86 patients with gastric cancer who had not previously received treatment. The average age of the patients was 62 years (61.9 ± 1.2). Depending on the histotype of the tumor, the patients were divided into six groups: 1) well- and moderately-differentiated adenocarcinoma (G1-2) – 24 people, 16 men and 8 women; 2) low-differentiated adenocarcinoma (G3) – 15 patients, 10 men and 5 women; 3) signet ring cell carcinoma (SRCC) – 19 people, 10 men and 9 women; 4) combined gastric lesions (CGL) – adenocarcinoma with the presence of signet ring cell fragments – 15 patients, 14 men and 1 woman; 5) patients with undifferentiated cells and signet ring cell fragments (G4 + SRCC) – 5 people, 3 men and 2 women; 6) patients with undifferentiated cells in gastric adenocarcinoma (G4 + G2–3) – 8 people, 6 men and 2 women. In the G1-2 group, moderately differentiated adenocarcinoma was detected in 92% of cases. In addition, the results of the study were separately analyzed in patients with serosal invasion and the spread to adjacent structures (T4 according to the TNM classification of malignant tumors) and in patients with stage IV cancer. All studied indicators in the blood of patients were compared with the corresponding values in a group of healthy men and women of comparable age without cancer (the donor group consisted of 20 people). All patients gave an informed consent for the use of biological material for scientific research.

Red blood cells were obtained by centrifuging heparinized blood for 15 min at 1 500 rpm, followed by three times washing them with physiological saline, buffered with phosphate buffer (pH 7.4), and centrifuging at 3 000 rpm for 10 min, at 4 °C. In the red blood

cells of patients with gastric cancer, the structural and functional parameters of the membranes were evaluated using a pyrene-based fluorescence probe (C16H10, M = 202.3; Serva, USA), determining the fluidity of the membranes in the region of the lipid bilayer and protein-lipid contacts, the polarity of the lipid bilayer and the protein immersion into the lipid matrix of the membrane according to the method of Yu.A. Vladimirova and G.E. Dobretsova (1980) [7] on the Fluorat-02-Panorama spectrofluorimeter (Russia). The method for determining the fluidity of membranes is based on the ability of a pyrene-based fluorescent probe to excimerize in a non-polar medium. The fluidity of the lipid bilayer of membranes is directly proportional to the excimerization coefficient of pyrene Fe / Fm, determined by the ratio of the fluorescence peak of the pyrene excimer Fe (in relative fluorescence units at emission wavelength λemi. = 470 nm) to the fluorescence peak of the pyrene monomer Fm (in relative fluorescence units at λemi. = 393 nm) with excitation wavelength $\lambda_{exc.}$ = 334 nm. The fluidity of protein-lipid contacts was determined by the ratio of the fluorescence intensity of the pyrene excimer to the pyrene monomer at $\lambda_{exc.}$ = 282 nm. The method is based on the ability of pyrene to intercept the energy of absorbed light from aromatic protein residues within a distance called the Förster radius. The polarity of the lipid phase of the membranes was evaluated by the ratio of the fluorescence intensities of the two monomeric forms F_{372} / F_{393} in the thin structure of the pyrene spectrum at $\lambda_{exc.}$ = 334 nm. The degree of immersion of proteins in the lipid bilayer was determined by quenching of the fluorescence of aromatic amino acid residues (tyrosine and tryptophan) following non-radiative energy transfer to the pyrene molecule. To measure protein immersion, the erythrocyte suspension was fluorimetric at $\lambda_{exc.}$ = 282 nm and λemi. = 330 nm. Then, after incubation with pyrene for 1 minute, the fluorescence intensity of the sample was measured again. The height of the fluorescence peak in this case (F) was less than in the first measurement (F_0) due to quenching of tryptophan fluorescence by pyrene. We determined the efficiency of energy transfer from tryptophanyls to pyrene that was equal to the value of $F_0 - F$.

Statistical processing of the results was carried out using the Statistica 6.0 software package and Student's t-test for two independent samples. The differences were considered statistically significant at $p < 0.05$, and at $0.1 > p > 0.05$ a trend towards statistical significance was observed. The samples were preliminarily checked for compliance with the normal distribution according to the Shapiro – Wilk W-test and Kolmogorov – Smirnov criterion. The data were presented as $M \pm m$, where M is the sample mean, and m is the error of the mean.

RESULTS

An examination of the state of erythrocyte membranes showed an increase in their fluidity in all groups of patients with gastric cancer, compared with the level both in the zone of protein-lipid contacts and in the lipid bilayer in the donors (table). Only in G1–2 patients fluidity was elevated only in the zone of protein-lipid contacts – by 31.1%. In patients with low-grade adenocarcinoma, this indicator in the zone of protein-lipid contacts increased by 52.3% compared to the donor level, in the lipid bilayer – by 36.6%, which was 30.2% higher than in G1–2 patients (p = 0.054). An increase in the adenocarcinoma extent (T4) did not significantly affect the level of fluidity – it was elevated in the zone of protein-lipid contacts by 38.5% ($p < 0.001$), and in the lipid bilayer there was a trend towards its increase by 19.5%. The most pronounced increase in erythrocyte membrane fluidity was detected in patients with adenocarcinoma with undifferentiated cells (G4 + G2-–3): by 93.8% in the zone of protein-lipid contacts and by 54.1% in the lipid bilayer in comparison with healthy people, which was statistically significantly higher than the level in G1–2 patients – by 47.8% and 46.9%, respectively ($p < 0.05$). In patients with SRCC and combined gastric lesions, fluidity was elevated in the zone of protein-lipid contacts by 36–38% and in the lipid bilayer by 17.2–21.5%. In the presence of undifferentiated cells in patients with SRCC, an increase in fluidity was 51.9% and 34%, respectively, as opposed to healthy people. The highest increase in fluidity of the lipid membrane bilayer was found in patients with stage IV disease – by 62.4%, while in the zone of protein-lipid contacts the indicator increased by 32.3%.

An increase in the fluidity of membranes in almost all groups was accompanied by an increase in the polarity of their lipid phase. A statistically significant increase in the polarity was detected in patients with moderately-differentiated adenocarcinoma – by 3.9%, with the advanced form of adenocarcinoma (T4) – by 4.6%, with combined lesions – by 5.1%. The greatest increase in polarity was recorded in patients with adenocarcinoma with the presence of undifferentiated cells – by 7.1% and with stage IV disease – by 7.8%.

Table

Structural and functional indicators of erythrocyte membranes in patients with different histotypes and stages of gastric cancer, relative fluorescence units, $M \pm m$

Groups of patients depending on histology and stage	Membrane fluidity in the zone of		Polarity of the membrane lipid phase	Protein immersion in the lipid matrix
	protein-lipid contacts	lipid bilayer		
Healthy people, $n = 20$	0.405 ± 0.010	0.303 ± 0.015	1.422 ± 0.013	0.203 ± 0.005
Adenocarcinoma G1–2, $n = 24$	0.531 ± 0.028 $p < 0.001$	0.318 ± 0.017 $p_{IVSt} = 0.011$	1.477 ± 0.017 $p = 0.013$	0.204 ± 0.012 $p_{IVSt} = 0.050$
Adenocarcinoma G3, $n = 15$	0.617 ± 0.057 $p < 0.001$	0.414 ± 0.053 $p = 0.017$ $p_1 = 0.054$	1.44 8 ± 0.024	0.199 ± 0.017 $p_{IV} = 0.082$
Signet ring cell carcinoma (SRCC), $n = 19$	0.551 ± 0.021 $p < 0.001$	0.355 ± 0.016 $p = 0.020$ $p_{IVSt} = 0.040$	1.454 ± 0.018 $p_{IVSt} = 0.078$	0.198 ± 0.017 $p_{IVSt} = 0.086$
Combined gastric lesions (G2–3+ SRCC), $n = 15$	0.559 ± 0.027 $p < 0.001$	0.368 ± 0.038 $p = 0.066$	1.494 ± 0.018 $p = 0.002$	0.231 ± 0.016 $p = 0.077$
Presence of undifferentiated cells and signet ring cell fragments (G4+ SRCC), $n = 5$	0.615 ± 0.08 $p < 0.001$	0.406 ± 0.107 $p = 0.071$	1.457 ± 0.024	0.219 ± 0.032
Presence of undifferentiated cells in adenocarcinoma (G4+G2–3), $n = 8$	0.785 ± 0.155 $p < 0.001$ $p_1 = 0.019$ $p_{IVSt} = 0.065$	0.467 ± 0.105 $p = 0.014$ $p_1 = 0.037$	1.523 ± 0.033 $p = 0.002$ $p_2 = 0.084$	0.171 ± 0.029 p = 0.035 $p_{IVSt} = 0.037$
Adenocarcinoma T4, $n = 17$	0.561 ± 0.035 $p < 0.001$	0.362 ± 0.033 $p = 0.074$	1.487 ± 0.021 $p = 0.010$	0.223 ± 0.019
IV stage, $n = 4$	0.536 ± 0.035 $p < 0.001$	0.492 ± 0.125 $p = 0.003$	1.533 ± 0.037 $p = 0.003$	0.274 ± 0.049 $p = 0.013$

Note. The data are presented as $M \pm m$, where M is the sample mean; m is the error of the mean. The statistical significance of the differences: p – relative to the group of healthy people, p_1 – relative to the group of patients with adenocarcinoma G1–2, p_2 – relative to the group of patients with adenocarcinoma G3, p_{IVSt} – relative to patients with stage IV disease.

Interestingly, in patients with gastric cancer, a statistically significant increase in protein immersion into the lipid matrix of erythrocyte membranes was detected only at stage IV disease (by 35%, $p = 0.015$), and there was also a trend towards a significant increase in patients with combined lesions (by 13.8%, $p = 0.077$). Patients with undifferentiated cells in gastric adenocarcinoma showed a decrease in protein immersion (by 15.8%, $p = 0.035$). According to the data obtained at Rostov Research Institute of Oncology earlier, this indicator turned out to be the most labile and drastically increased at most localizations of the malignant process; and its changes often correlated with the state of patients and the effectiveness of therapy. Significant changes in the immersion of proteins in the lipid matrix of blood cell membranes were detected in gynecological cancers, breast cancer, lung cancer, oropharyngeal cancer, and malignant brain lesions [13, 14]. In patients with breast cancer with high efficacy of chemotherapy, normalization of initially increased protein immersion in the lipid matrix of erythrocyte and lymphocyte membranes was observed in the absence of positive changes in patients with tumor stabilization [15]. Therefore, in gastric cancer, the change in protein immersion in the lipid matrix of erythrocyte membranes differed from what was observed in other cancers.

Separately, it is necessary to dwell on the indicators of erythrocyte membranes in patients with stage IV gastric lesions. In these patients, the increase in the fluidity was more pronounced in the lipid bilayer – 62.4%, than in the zone of protein-lipid contacts – 32.3%, while in all other groups of patients the increase in fluidity in the zone of protein-lipid contacts was more pronounced. The maximum increase in the fluidity of the lipid bilayer among all groups corresponded to the largest increase in the polarity of the lipid phase of the membranes and in the immersion of proteins in the lipid matrix. The fluidity of the lipid bilayer in patients with stage IV lesion was significantly higher than in patients with adenocarcinoma G1–2 and SRCC (54.7% and 38.6%, respectively). A trend towards statistical significance of higher levels of polarity in these patients relative to patients with SRCC (by 5.4%) and immersion relative to patients with

adenocarcinoma G1–2, G3, and SRCC (by 34.3%, 37.7%, and 38.4%, respectively) was revealed. Statistically significant excess of protein immersion was observed in patients with stage IV disease, as opposed to a reduced level of this indicator in patients with G4 + G2–3 (by 60.2%), with a trend towards a significant decrease in the fluidity of protein-lipid contacts (by 31.7%) relative to the same group.

DISCUSSION

According to the data obtained, the majority of examined patients with gastric cancer were characterized by an increase in the erythrocyte membrane fluidity which was more pronounced in the zone of protein-lipid contacts, but also observed in the lipid bilayer. The greatest changes were revealed in the presence of undifferentiated cells in stomach tumors. In this case, an increase in the fluidity of erythrocyte membranes was observed both in gastric adenocarcinoma and in SRCC. In adenocarcinoma, it was also accompanied by a more pronounced increase in the polarity of the lipid phase of the membranes. And only the G4 + G2–3 group of patients was characterized by a statistically significant decrease in the immersion of proteins in the membrane lipid matrix.

The fluidity is known to characterize the ability of membranes to respond to disorders occurring in the body, including the development of a malignant process [16]. The reason for the increase in the fluidity of the lipid bilayer may be an increase in the free radical oxidation of membrane lipids, their interaction with lipid peroxidation products, and a change in the lipid composition of membranes [3, 17]. The main factors determining the fluidity of membranes include the content of cholesterol, which contributes to a more stringent ordering of the lipid bilayer, and the content of unsaturated lipids, which increase the fluidity and permeability of the membranes [2]. Since membrane lipids form the environment for the functioning of membrane proteins, a change in the structure of the lipid bilayer leads to a violation of the conformation of proteins associated with it. The intensification of free radical oxidation of membrane proteins leads to a change in their tertiary structure and an increase in protein aggregation or fragmentation, which contributes to an increase in fluidity in the zone of protein-lipid contacts, immersion of proteins in the lipid matrix and, as a result, a decrease in their functional capabilities, including the receptor apparatus [5]. It was shown that membrane areas (domains) enriched in cholesterol and sphingolipids, with epidermal growth factor receptors (EGFs), estrogens, and other receptors built into them, participate in cell proliferation, while a ceramide-enriched domain with built-in Fas receptors (CD95) and TNF-R1 triggers caspases and promotes apoptosis [2]. Changes in lipid domains and proteins within the domain can be involved in malignant transformation, uncontrolled growth, invasiveness and metastasis [18], and also affect the state of antioxidant and proteolytic systems that play an important role in cancer [19, 20].

The functional importance of changes in the protein component of membranes in malignant pathology is indirectly indicated by high frequency of increased fluidity of the membranes in the zone of protein-lipid contacts revealed in red blood cells of patients with gastric cancer. Our results on the intensification of lipid peroxidation in blood plasma and erythrocytes of patients with gastric cancer are consistent with the activation of free radical oxidation as one of the reasons for the increase in the fluidity of erythrocyte membranes. Herewith, the level of lipid peroxidation products and the degree of violation of conjugation of erythrocyte antioxidant enzymes increased with a decrease in the differentiation of adenocarcinoma, especially in the presence of undifferentiated cells in the tumor [21].

An analysis of the changes in the properties of membranes that occur in cancer led to the conclusion that an increase in the fluidity of the membranes of tumor cells promotes metastasis, and a decrease in the fluidity leads to the development of multidrug resistance [2]. Our data indicate that functionally significant structural features are characteristic not only for tumor cells. Changes in the fluidity, polarity of the lipid bilayer and the immersion of proteins in it also occur in erythrocyte membranes, depending on the histological characteristics of the tumor and types and stages of cancer. Thus, the results obtained in the study of erythrocyte membranes in gastric cancer are consistent with the opinion expressed by foreign researchers [2, 22, 23] that in-depth studies of cell membranes and their lipid composition, that is lipidomics of various types and stages of cancer, as well as modification of membrane components provide great opportunities for treatment and prevention of cancer and will be more often used in the coming years as markers of prognosis and progression in malignant pathologies.

CONCLUSION

An increase in the fluidity of erythrocyte membranes in the zone of protein-lipid contacts and lipid bilayer,

as a rule, is accompanied by a small, but statistically significant increase in the polarity of the lipid phase of the membranes. It is typical for patients with gastric cancer and increases with a decrease in the degree of tumor differentiation. The maximum increase in the fluidity and polarity of the lipid bilayer, accompanied by a significant increase in the immersion of proteins in the lipid matrix, was revealed in patients with stage IV tumors. The data obtained indicate that changes in the structural and, therefore, functional properties of membranes that are typical not only of tumor cells, but also of blood cells, can contribute to the development of the tumor process and can be used as prognostic markers of disease progression and treatment success.

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Authors contribution

Goroshinskaya I.A. – analysis and interpretation of the results and literature data, statistical processing of the results, drafting and design of the article. Nemashkalova L.A. – determination of membrane parameters, participation in the selection of literature. Frantsiyants E.M. – final approval of the manuscript for publication. Surikova E.I. – analysis of clinical indicators of patients for their division into groups. Medvedeva D.E. – collection of the material for research and provision of information on patients. Maslov A.A. – diagnosis, determination of a treatment plan for patients included in the study.

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Pathogenesis of anemia in pregnant women with gestational diabetes mellitus

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ABSTRACT

The aim of the research was to establish the role of inflammation mediators and iron metabolism in the pathogenesis of various types of anemic syndrome in pregnant women with gestational diabetes mellitus (GDM).

Materials and methods. 32 pregnant patients with GDM were examined; 14 of them had iron deficiency anemia, 18 – anemia of chronic diseases. The enzyme-linked immunosorbent assay was used to determine the concentration of IL-6, hepcidin and a soluble receptor for transferrin in the blood serum of pregnant women, the concentrations of C-reactive protein and transferrin were determined with the method of turbidimetry.

Results. It was shown that women with GDM had higher IL-6 level compared to healthy pregnant women, and the concentration of IL-6 did not depend on the type of anemic syndrome. The C-reactive protein concentration was higher in patients with GDM and anemia of chronic diseases than in healthy pregnant women or in pregnant women with iron deficiency anemia. An analysis of iron metabolism markers in pregnant women with GDM established that patients with anemia of chronic diseases had higher hepcidin levels than women with iron deficiency anemia or healthy pregnant women.

Conclusions. We established the heterogeneity of the anemic syndrome in pregnancy complicated by GDM. It was confirmed that GDM was accompanied by subclinical inflammation, which was more pronounced in anemia of chronic diseases. The research showed that the mechanism of development of anemia of chronic diseases involving the hepcidin protein was also realized in GDM, characterized by subclinical inflammation. The results indicate the importance of establishing the type of the anemic syndrome in pregnant women with GDM for effective therapeutic follow-up.

Key words: pregnancy, anemia of chronic diseases, gestational diabetes mellitus, inflammation, hepcidin.

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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 Siberian State Medical University (Protocol No. 3431, 2013).

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Патогенез анемического синдрома у беременных с гестационным сахарным диабетом

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РЕЗЮМЕ

Цель. Установить роль медиаторов воспаления и метаболизма железа в патогенезе различных видов анемического синдрома у беременных с гестационным сахарным диабетом (ГСД).

Материалы и методы. Проведено обследование 32 беременных с ГСД, из которых 14 пациенток имели железодефицитную анемию, а 18 – анемию хронических заболеваний. В сыворотке крови беременных методом иммуноферментного анализа определяли концентрацию интерлейкина 6, гепсидина, растворимого рецептора к трансферрину, методом турбидиметрии – концентрацию С-реактивного белка и трансферрина.

Результаты. У беременных с ГСД концентрация интерлейкина 6 повышена в сравнении с его уровнем у здоровых беременных и не зависит от вида анемического синдрома. У беременных с ГСД, имевших анемию хронических заболеваний, концентрация в крови С-реактивного белка превышала таковую у здоровых беременных и беременных с железодефицитной анемией. Анализ содержания в крови у беременных с ГСД маркеров метаболизма железа показал, что у женщин с анемией хронических заболеваний концентрация гепсидина значительно выше, чем у пациенток с железодефицитной анемией и здоровых беременных.

Заключение. Установлена гетерогенность анемического синдрома при беременности, отягощенной ГСД: истинную железодефицитную анемию имели менее половины пациенток, в то время как у большей части женщин анемия была связана с внутриклеточной блокадой железа. Подтверждено, что ГСД сопровождается развитием субклинического воспаления, более выраженного при анемии хронических заболеваний. Показано, что механизм развития анемии хронических заболеваний с участием белка гепсидина реализуется и при ГСД, характеризующимся субклиническим воспалением. Полученные результаты указывают на важность установления вида анемического синдрома у беременных с ГСД с целью эффективного терапевтического сопровождения пациенток.

Ключевые слова: беременность, анемия хронических заболеваний, гестационный сахарный диабет, воспаление, гепсидин.

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INTRODUCTION

Carbohydrate metabolism disorder and anemic syndrome are some of the most common extragenital diseases in pregnant women. According to the World Health Organization (2014), in most cases, anemia in pregnant women is iron deficiency anemia, and its frequency in developed countries reaches up to 20–25%, in Russia – up to 35–43%. However, due to glucose toxicity and lipotoxicity, anemia of chronic diseases resistant to iron therapy is very likely to develop in pregnant women with carbohydrate metabolism disorders. High prevalence of the anemic syndrome together with its heterogeneity (in the presence of a concomitant pathology) in pregnant women and possible complications (increased perinatal mortality, fetal growth retardation, intrauterine hypoxia, neonatal asphyxia, weak labor, poor tolerance of blood loss, etc.) make both studying the anemia pathogenesis and improving the methods of its laboratory diagnosis essensial [1].

The aim of the study was to establish the role of inflammation mediators and iron metabolism in the pathogenesis of various types of the anemic syndrome in pregnant women with gestational diabetes mellitus.

MATERIALS AND METHODS

The study included 32 pregnant women with gestational diabetes mellitus (GDM) followed up by an endocrinologist at Regional Perinatal Center (Tomsk). Gestational diabetes mellitus was diagnosed in the first trimester of pregnancy during the first biochemical screening according to the diagnostic criteria in compliance with the Russian National Consensus Statement on gestational diabetes mellitus: diagnosis, treatment and postnatal care, 2012. In all patients, GDM was treated with diet therapy, without the use of insulin preparations. The anemic syndrome was diagnosed and specified following complex analysis of laboratory data. The hematology profile (red blood cells, reticulocytes, hemoglobin) (using a 5-diff hematology analyzer Sysmex xs-1000i, Sysmex, Japan) and blood serum chemistry (serum iron, total iron binding capacity, serum ferritin) (using a Cobas analyzer with 311, Roche, Germany) were studied. A decrease in hemoglobin concentration lower than 110 g/l was considered as the anemic syndrome in pregnant women. Iron deficiency anemia was verified in case of a decrease in blood levels of serum iron and ferritin, in combination with increased total iron binding capacity. Anemia of chronic diseases was established in case of reduced total iron binding capacity and serum iron concentration, but normal or increased ferritin concentration. All pregnant women were divided into two groups: iron deficiency anemia (IDA) – 14 patients, and anemia of chronic diseases (ACD) – 18 patients.

At the time of the study, all patients were in the second trimester of pregnancy, aged 23 – 44 years. The control group consisted of 12 healthy pregnant women of comparable age. Exclusion criteria were the use of iron preparations, acute infections or exacerbation of chronic infections, purulent necrotic diseases, allergic diseases (bronchial asthma, atopic dermatitis, etc.) in the medical history or at the time of the screening, nephritis of any etiology, psoriasis, and refusal to participate in the study. The study met ethical standards; all individuals participating in the study signed an informed consent.

The study material was venous blood serum taken in the morning before ingestion from the ulnar vein in an amount of 10 ml into a BD Vacutainer vacuum tube with a coagulation activator with silica particles (Becton Dickinson, USA). The concentrations of IL-6 (pg/ml) (Vector-Best, Russia), hepcidin (ng/ml) (MyBioSource, USA) and soluble transferrin receptor (sTfR) (nmol/l) (R&D Systems, USA) were determined by the serum-linked immunosorbent assay in blood serum according to the instructions of reagent kit manufacturers. The results were read using an automatic photometer for Sunrise microplates (Tecan, Austria) at a wavelength of 450 nm. The concentration of the studied markers was determined by a standard calibration curve. The concentrations of C-reactive protein (CRP) (mg/l) (at a wavelength of 552 nm) and transferrin (g/l) (at a wavelength of 570 nm) were determined in the blood serum by the turbidimetric method using a Cobas c311 analyzer (Roche, Germany).

Statistical processing of the obtained data was performed using the SPSS Statistics 18 software package. The distribution normality was checked using the Shapiro – Wilk criterion. The threshold level of statistical significance of differences was 0.05.

The data obtained did not obey the normal distribution law and were presented as the median and interquartile range (*Me*, Q_{25}–Q_{75}). The significance of differences between independent comparison groups was established using the Kruskal – Wallis criteria with the Bonferoni correction for the three study groups, and using the Mann – Whitney test for two groups. The correlation between the studied parameters was evaluated using the Spearman's test for nonparametric data.

RESULTS

It was found that the concentration of IL-6 increased in pregnant women with GDM, ($p < 0.05$) compared to healthy pregnant women and did not depend on the type of the anemic syndrome. CRP level was also higher in patients with GDM and ACD compared to patients with IDA and healthy pregnant women ($p < 0.05$ for both cases) (Table 1).

The analysis of iron metabolism markers in women with GDM showed that patients with ACD had higher hepcidin concentrations, than those with IDA ($p < 0.05$) and healthy pregnant women ($p < 0.05$). An average positive linear relationship was found between the concentrations of hepcidin and CRP ($r = 0.61$; $p < 0.05$) in the blood serum of patients with gestational diabetes mellitus and anemia of chronic diseases. There were no differences in transferrin and sTfR concentrations in IDA and ACD patients ($p > 0.05$ in all cases) (Table).

Table

The concentration of inflammatory and iron metabolism markers in the serum of pregnant women with gestational diabetes mellitus, taking into account the type of anemia, *Me* (Q_{25}–Q_{75})

Parameter	Pregnant women without gestational diabetes mellitus and anemia ($n = 12$)	Pregnant women with gestational diabetes mellitus	
		Pregnant women with iron deficiency anemia ($n = 14$)	Pregnant women with anemia of chronic disease ($n = 18$)
IL-6 (pg/ml)	0.5 (0–1.0)	2.8 (2.6–2.8)*	2.7 (1.8–3.0)*
C-reactive protein (mg/l)	1.33 (1.0–1.65)	2.82 (1.85–3.29)	8.79 (6.32–10.12) * **
Hepcidin (ng/ml)	5.55 (0–11.1)	7.1 (3.4–11.7)	12.2 (11.6–14.6)* **
sTfR (nmol/l)	20.15 (5.94–34.36)	34.79 (23.92–38.85)	38.41 (24.5–42.47)
Transferrin (g/l)	–	4.03 (3.65–4.39)	4.05 (4.05–4.15)

* the differences are significant against similar indexes in pregnant women without gestational diabetes mellitus and anemia ($p < 0.05$); ** against pregnant women with iron deficiency anemia.

However, we found a strong positive linear relationship between the increasing concentrations of transferrin and its soluble receptor ($r = 0.84$; $p < 0.05$) in the blood serum of patients with GDM and IDA, which reflects the classical concept of IDA pathogenesis.

DISCUSSION

Already at the stage of patients' stratification into clinical examination groups, the study showed that in almost half of cases anemia in pregnant women with GDM was not iron deficiency anemia and, therefore, could not be treated by iron preparations. The heterogeneity of the structure of the anemic syndrome in individuals with type 1 diabetes mellitus (both in pregnant and non-pregnant patients with type 1 diabetes mellitus) has been confirmed by other studies [2, 3]. Thus, it is necessary to clearly differentiate anemia of chronic diseases and iron deficiency anemia in pregnant women with impaired carbohydrate metabolism.

It was established that in anemia of chronic diseases, the key role in the activation of hepcidin synthesis belongs to the group of proinflammatory cytokines – IL-1, IL-6, TNFα [4], and especially to IL-6 [5]. The main function of hepcidin, in turn, is to block the action of the iron carrier protein, ferroportin, as a result of which iron is disrupted from macrophages, enterocytes, placenta, and other cells, leading to hypoferremia [6]. Thus, the pathogenesis of anemia of chronic diseases is associated with an excess of IL-6 production, activation of hepcidin synthesis and a decrease in the availability of iron for erythropoiesis. This study, as well as our previous works [7, 8], indicated the presence of an inflammatory process in GDM, since the concentration of IL-6

in the blood serum of women with GDM was higher than during physiological pregnancy. It should be noted that the concentration of IL-6 in pregnant women with GDM had comparable values for various types of the anemic syndrome. Moreover, a significant increase in hepcidin concentration was observed only in pregnant women with anemia of chronic diseases. This indicates the presence of additional factors that stimulate hepcidin production and contribute to the development of anemia of chronic diseases in GDM. The most sensitive clinical and laboratory indicator of inflammation is C-reactive protein. The highest concentration of C-reactive protein was recorded in the blood serum of pregnant women with GDM who had anemia of chronic diseases, which indicates a greater activation of inflammation in these patients, compared with pregnant women suffering from iron deficiency anemia. Therefore, the mechanism of development of anemia of chronic diseases involving hepcidin, IL-6 and C-reactive protein is also realized in subclinical inflammation accompanying gestational diabetes mellitus.

Transferrin and sTfR are considered as differential diagnostic markers of IDA and ACD. It is stated that patients with anemia of chronic diseases have normal or reduced concentrations of transferrin and sTfR in the blood serum, while in patients with iron deficiency anemia the concentrations of these markers increase [9, 10]. However, the content of transferrin and sTfR in the blood reflects both the amount of iron deposited in the body and the activity of erythropoiesis [11, 12], which increases by the end of the first trimester of pregnancy. Thus, the informative value of these tests after 12 weeks of pregnancy is doubtful.

This study confirmed the limitation of using transferrin and its soluble receptor as indicators of ACD verification in pregnant women with GDM.

CONCLUSION

Gestational diabetes mellitus is accompanied by subclinical inflammation, which is more pronounced in anemia of chronic diseases, as opposed to iron deficiency anemia. The mechanism of development of anemia of chronic diseases in GDM involves the hepcidin protein. The heterogeneity of the anemic syndrome in pregnancy complicated by GDM was established: less than half of patients had true iron deficiency anemia, while in most women anemia was associated with intracellular iron blockade. The results indicate the importance of correct differential diagnosis of the anemic syndrome in pregnant women with GDM.

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Authors contribution

Zima A.P. – conception and design, final approval of the manuscript for publication. Prokhorenko T.S. – carrying out of experimental work, analysis and interpretation of data. Saprina T.V. – analysis and interpretation of data. Musina N.N. – carrying out of experimental work, processing of data. Novitsky V.V. – substantiation and critical revision of the manuscript for important intellectual content. Baykov A.N. – interpretation of the results, drafting of the manuscript.

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Association of serotonin 2C receptor gene polymorphism with depression and quality of life indicators in patients before coronary artery bypass grafting

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ABSTRACT

The aim was to study the association of the rs6318 polymorphism of the *HTR2C* gene with the level of depression and quality of life in patients undergoing coronary artery bypass grafting (CABG).

Materials and methods. A total of 116 patients with coronary artery disease (CAD) (age 60 [57; 65] years) were examined before CABG. Depression was assessed in all patients in the preoperative period using the Beck Depression Inventory (BDI). In addition, the quality of life was measured in all patients using the SF-36 questionnaire. Blood samples were collected for the subsequent polymerase chain reaction-based genotyping to detect the rs6318 polymorphism of the *HTR2C* gene. Statistical analysis was performed using the STATISTICA 10.0 software package (StatSoft Inc., USA). The value of $p < 0.05$ was considered statistically significant.

Results. No significant differences were found in the associations between different genotypes of the HTR2C gene and depression levels. However, certain trends have been established ($p = 0.1$). Thus, the pairwise comparison of different genotypes reported that carriers of the CC genotype had higher BDI scores (12 [8; 19]), whereas carriers of the CG genotype ($p = 0.07$) and GG genotype ($p = 0.08$) had lower BDI scores (3.5 [2; 5] and 8 [0; 25], respectively). The quality of life among carriers of the CC, CG and GG genotypes did not differ significantly. Nevertheless, the median values of almost all indicators (GH, PF, RE, VT) were lower in carriers of the CC genotype. Carriers of the CC genotype suffered more from pain limiting their daily activities than carriers of the GG genotype ($p = 0.04$). Homozygous C allele carriers demonstrated poorer mental health than heterozygous carriers (56 [40; 64] vs. 82 [72; 92], $p = 0.04$).

Conclusions. Reliable associations of different genotypes of the rs6318 polymorphism of the *HTR2C* gene with the quality of life parameters have been found in patients with coronary artery disease.

Key words: coronary artery disease, coronary artery bypass grafting, depression, quality of life

Conflict of interest. The authors declare no 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. The study protocol and design were approved by the local Ethics Committee of the Research Institute for Complex Issues of Cardiovascular Diseases (Protocol No. 20 of 25.01.2011).

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Ассоциация полиморфизма гена рецептора серотонина 2С с депрессией и показателями качества жизни у пациентов перед операцией коронарного шунтирования

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РЕЗЮМЕ

Цель. Изучить связи генетического полиморфизма rs6318 гена *HTR2C* с уровнем депрессии и качеством жизни у пациентов перед коронарным шунтированием (КШ).

Материалы и методы. Обследованы 116 пациентов с ишемической болезнью сердца (ИБС) перед КШ (возраст 60 [57; 65] лет). В предоперационном периоде проводилась оценка депрессии с помощью шкалы Бека, качества жизни с применением опросника SF-36, а также проводился забор крови с последующим генотипированием полиморфизма rs6318 *HTR2C* методом полимеразно-цепной реакции. Статистическая обработка осуществлялась с использованием пакета программ Statistica 10.0 (StatSoft Inc., США). Во всех случаях нулевую гипотезу отвергали при $p < 0,05$.

Результаты. При анализе связи различных генотипов гена *HTR2C* с уровнем депрессии значимых различий не найдено, однако установлены определенные тенденции ($p = 0,1$). Так, при попарном сравнении различных генотипов обнаружено, что у носителей генотипа CC балл по шкале Бека был выше и составил 12 [8; 19], тогда как у носителей генотипов CG ($p = 0,07$) и GG ($p = 0,08$) он был ниже и составил 3,5 [2; 5] и 8 [0; 25] соответственно. Показатели качества жизни у носителей генотипов CC, CG и GG значимо не различались, однако, значение медианы практически по всем показателям опросника (GH, PF, RE, VT) было ниже у носителей генотипа CC. У носителей генотипа CC боль ограничивала их повседневную деятельность больше, чем у носителей генотипа GG ($p = 0,04$). У гомозигот по аллелю *C* уровень психического здоровья было также ниже, чем у гетерозигот (56 [40; 64] против 82 [72; 92], $p = 0,04$).

Заключение. В настоящем исследовании обнаружены статистически значимые связи различных генотипов полиморфизма rs6318 гена *HTR2C* у пациентов с ИБС с показателями качества жизни.

Ключевые слова: ишемическая болезнь сердца, коронарное шунтирование, депрессия, качество жизни.

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

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Соответствие принципам этики. Всеми пациентами было подписано добровольное информированное согласие. Исследование было одобрено локальным этическим комитетом НИИ КПССЗ (протокол № 20 от 25.01.2011).

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INTRODUCTION

Effectiveness of coronary artery bypass grafting (CABG) for treating patients with coronary artery disease (CAD) has already been proven and is of no doubt [1]. However, despite all recent advances in the CABG surgical strategies, anesthesia and pre- and postoperative management, the proportion of patients becoming disabled after CABG remains very high [2]. However, CABG is aimed at improving the quality of life (QoL), not worsening it. There are

many reasons that might provoke this paradox: a lack of cardiac rehabilitation programs at each phase of rehabilitation, low patients' awareness of cardiac rehabilitation benefits [3], limited ability of patients to participate in these programs, etc. Patients' unwillingness to participate may be explained by depression and anxiety before and after CABG. In addition, cardiac surgery itself is considered as a significant stressful factor for patients [4]. The prevalence of depression and severe anxiety among patients before cardiac surgery ranges from 30 to 40% [5].

Over the past decades, it has been proven that personality traits are largely dependent on genetic factors [6]. Since the serotonergic system is actively involved in the pathogenesis of depression [7], the genes associated with it are currently being actively studied. Particular attention is focused on the serotonin 2c receptor gene (HTR2C), located on the chromosome Xq24 site and responsible for social behavior and cognition.

HT2C receptors are found in the striatum, choroid plexus, cerebral cortex, hippocampus, and substantia nigra. 2C receptors have been shown to control the release of other neurotransmitters, such as norepinephrine and dopamine. HT2C receptors are involved in the regulation of mood, anxiety, sexual functions, sleep, appetite, and the cardiovascular system [8].

Cys23Ser (rs6318) is one of the most well-studied HTR2C gene polymorphisms. The replacement of guanine by cytosine results in the amino acid replacement, and cysteine is then replaced by serine. Binding of the receptors in CC genotype carriers is two times weaker than in homozygous GG carriers. Thus, CC carriers are supposed to be more prone to the onset of depressive disorders [9, 10]. A number of studies have reported the association between this polymorphism and suicidal behavior [11], alcoholism, bipolar mental disorders, schizophrenia [12], and major depressive disorders [13].

However, evidences on the contributive role of this polymorphism in the development of anxiety and depression in somatic patients are limited.

Our study is aimed at determining the relationship of HTR2C (rs6318) gene polymorphism with the level of depression and quality of life in patients undergoing preoperative management for CABG.

MATERIALS AND METHODS

116 patients with stable coronary artery disease undergoing preoperative management for on-pump CABG were enrolled in the study. Of those, 95 (82%) were men and 21 (18%) were women. The median age was 60 [57; 65] years. The exclusion criteria were the presence of severe somatic pathology (acute or chronic renal failure, liver failure, chronic lung diseases, thyroid diseases, autoimmune diseases) and refusal to participate in genotyping.

Two-to-seven days before CABG, all patients underwent depression screening using the Beck Depression Inventory and the quality of life assessment using the SF-36 questionnaire. Eight subscales were assessed (physical functioning (PF), role-physical functioning (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional functioning (RE) and mental health (MH). Blood sampling was performed at days 3–5 before CABG, followed by the genotyping of HTR2C (rs6318) polymorphism. DNA was isolated by phenol-chloroform extraction according to Maniatis et al. The concentration of obtained DNA was measured on the NanoDrop-2000 spectrophotometer. DNA quantification was performed using real-time polymerase chain reaction with the Taqman-probes (a 96-well plate). The Hardy – Weinberg equilibrium was met.

Clinical and demographic data of patients are presented in Table 1.

Half of the patients had myocardial infarction and arterial hypertension in their medical history and a quarter of patients had diabetes mellitus.

All patients had been receiving standard four-component CAD therapy (antiplatelet agents, beta-blockers, statins, ACE inhibitors or angiotensin receptor blockers) for 7 days before surgery.

Statistical analysis was performed using commercially available software package Statistica 10.0 (Statsoft Inc., USA). The data distribution was different from normal. The data are presented as absolute values and percentage, as well as the median and interquartile range. The Kruskal – Wallis test was used to measure the differences between the groups. A p value of ≤ 0.05 was considered statistically significant.

Table 1

Clinical and demographic data of patients before coronary artery bypass grafting

Parameter	Value
Age, years, *Me* [Q_1; Q_3]	60 [57; 65]
BMI, kg/m^2, *Me* [Q_1; Q_3]	28.5 [26.5; 30.7]
EuroScore 2, % , *Me* [Q_1; Q_3]	1.68 [1.27; 1.98]
Duration of coronary artery disease, years, *Me* [Q_1; Q_3]	4 [1;8]
Arterial hypertension, *n* (%)	95 (47)
Duration of arterial hypertension, years, *Me* [Q_1; Q_3]	10 [5; 15]
Angina pectoris, *n* (%): – 0; – I; – II; – III	 6 (5) 6 (5) 79 (68) 25 (22)
Heart failure (NYHA), *n* (%): – I; – II; – III	 12 (10) 95 (82) 9 (8)
Prior myocardial infarction, *n* (%)	64 (55)
Prior stroke, *n* (%)	6 (5)
History of type 2 diabetes mellitus, *n* (%)	29 (25)
Left ventricular ejection fraction, %, *Me* [Q_1; Q_3]	60 [51; 65]

Note. BMI – body mass index, NYHA – New York Heart Association.

RESULTS

At the first stage of the study, the prevalence of depression was assessed in the studied group of patients. According to the Beck Depression Inventory, most patients (64%) did not have depression (0–9 points), 20% of patients had minimal depression (10–15 points), 9% – mild (16–19 points) and 7% – moderate (20–29 points). There were no patients with severe depression (30–63 points) in the studied group. The distribution of patients depending on the genotype of rs6318 polymorphism is presented in Table 2.

Table 2

Depression level measured by the Beck Depression Inventory in the carriers of different genotypes of the rs6318 polymorphism of the *HTR2C* gene

Beck Depression Inventory, depression level	Genotype, *n* (%)		
	GG	CG	CC
None	68 (59)	2 (2)	3 (3)
Minimal	22 (19)	0 (0)	2 (1)
Mild	10 (8)	0 (0)	1 (1)
Moderate	7 (6)	0 (0)	1 (1)
Severe	0 (0)	0 (0)	0 (0)

The analysis of the relationships of different *HTR2C* genotypes with the level of depression measured by the Beck Depression Inventory did not show any significant differences. However, some trends were established (p = 0.1). The pairwise comparison of different genotypes reported that CC genotype carriers had higher BDI score of up to 12 [8; 19], while in CG genotype carriers (p = 0.07) and GG genotype carriers (p = 0.08) it was up to 3.5 [2; 5] and 8 [0; 25], respectively. Then, we analyzed the presence of the relationships between the quality of life and various genotypes of the *HTR2C* gene. There were no significant differences in PF, RP, GH, VT, SF, and RE between CC, CG and GG genotype carriers. The obtained data are presented in Table 3.

CC genotype carries reported lower mean score of GH, PF, RE, and VT, indicating the worst physical and mental health. The absence of reliable differences might be associated with a small sample size.

Table 3

The mean SF-36 score, depending on the different genotypes of polymorphism rs6318, *Me* [Q_1; Q_3]

SF-36 scale	*HTR2C* rs6318, *Me* [Q_1; Q_3]			*p*
	GG	CG	CC	
SF	50 [38; 50]	56.5 [50; 63]	50 [38; 63]	0.09
GH	50 [40; 62]	68.5 [55; 82]	40 [35; 52]	0.34
PF	55 [35; 80]	62.5 [30; 95]	50 [30; 60]	0.56
RP	0 [0; 50]	25 [0; 50]	25 [0; 50]	0.99
RE	34 [0;67]	83.5 [67; 100]	0 [0; 34]	0.42
VT	55 [40; 70]	67.5 [55; 80]	45 [30; 55]	0.36

Pain in CC genotype carriers interfered with their daily activities more than in GG genotype carriers (p = 0.04). The mean score was 41 [31; 62], and it was significantly lower than that of homozygotes for the G 51 allele [41; 74]. Moreover, homozygotes for the C allele had poorer mental health. Patients with this genotype had lower MH score than heterozygotes (p = 0.04): 56 [40; 64] vs. 82 [72; 92] (Fig. 1).

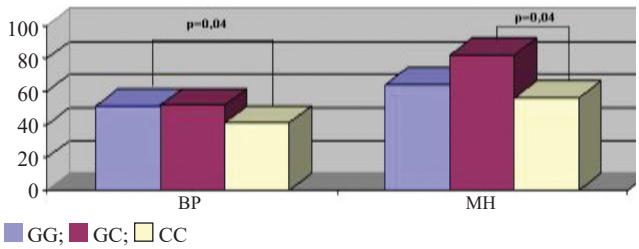

Figure 1. Distribution of mental health scores and bodily pain scores depending on the genotypes of the rs6318 polymorphism of the *HTR2C* gene

DISCUSSION

To date, the effects of depression and anxiety on the development and adverse course of coronary artery disease has been proven. The Framingham study has reported the correlation of depression with the onset of CAD [14].

Over the past decade, both pilot and large-scale studies have shown that not only depression, but also a high level of anxiety affect the course of coronary artery disease. Sumin et al. have found that CAD patients with polyvascular disease have higher depression and anxiety levels than patients with isolated coronary artery disease [15]. The Russian multicenter study COORDINATE performed in 37 cities from 2007 to 2009 demonstrated that a high level of anxiety in patients with arterial hypertension and coronary artery disease increases the risk of death during 1.5 years by 45% [16].

CAD patients undergoing CABG are a special group of patients. These patients undergo massive cardiac surgery with the extracorporeal circulation, while the surgery itself is aimed at improving their quality of life in the future and should not result in disability. Thus, this group of patients has long attracted the attention of researchers from the perspective of studying depression and anxiety and their cumulative impact on the postoperative period, as well as on the short- and long-term prognoses. The presence of anxiety in the preoperative period significantly increases the probability of death in the postoperative hospital period, as well as the development of atrial fibrillation, stroke, myocardial infarction and renal failure [17, 18].

The serotonergic system plays an important role in the regulation of social behavior. Given the fact that polymorphic loci of genes determine the activity of the product that they encode, various polymorphic variants of genes responsible for exchange of serotonin, including *HTR2C* gene, can cause the development of depression and high anxiety [19].

The rs6318 polymorphism of the *HTR2C* gene is located in the position affecting the coding region of the gene, and, therefore, may affect expression. It is hypothesized that CC genotype carriers synthesize the protein that may reduce affinity for serotonin [20].

Alfimova M.V. et al. have demonstrated that the level of anxiety in C allele carriers is higher than in G allele carriers of the rs6318 polymorphism of the *HTR2C* gene among patients with schizophrenia (n = 337) and mentally healthy individuals (n = 333) [12]. Levchuk et al. compared the distribution of genotypes of the rs6318 polymorphism in 22 men with depression and 29 somatically and mentally healthy men. They reported that the GG genotype in men with depression was significantly less common than in somatically healthy men [10].

We have not found any associations between the genotypes and depression. However, patients with the CC genotype have shown a higher level of depression according to the Beck Depression Inventory. The absence of statistically significant associations of any genotype with the presence of depression might be explained by the absence of depression in the majority of the studied patients (64%).

Quality of life indicators are a more subtle marker for anxiety-depressive disorders; they allow to identify a group of patients who are at high risk of adverse events in the postoperative period and those who will be less compliant with drug therapy and rehabilitation.

CC genotype carriers have demonstrated lower mean SF-36 score than other genotype carriers. Statistically significant differences were found for mental health and bodily pain. Similar findings were reported in the study of Golimbet et al. Out of 167 CAD patients, C allele carriers of the rs6318 polymorphism of the *HTR2C* gene were more likely to have a painful form of coronary artery disease and hostility, i.e. factors predisposing to the development of depression [21].

Thus, there is a close relationship between genetic polymorphisms and the development of depression. The impact of genes on the development of schizophrenia, suicidal behavior and major depressive disorders is widely studied both in the world and in Russia. However, the relationship of genes with subclinical depression and a high level of anxiety in patients with somatic pathology, including coronary artery disease, requires further detailed studies.

CONCLUSION

The obtained findings suggest the presence of the association between the rs6318 genetic polymorphism of the *HTR2C* gene and low quality of life in patients undergoing CABG, which can be considered as an

unfavorable prognostic factor in the postoperative period. This polymorphism seems to be promising in screening of patients prone to depression, giving the opportunity to early diagnose and timely prevent anxiety and depression (patient education, sessions with psychologists, and educational consultations with cardiologists).

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The experimental model of type 2 diabetes mellitus caused by a high-fat diet with low-dose streptozotocin in rats

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ABSTRACT

Aim. To develop a pathogenetically reasonable model of type 2 diabetes with marked peripheral insulin resistance and relative insulin deficiency in rats using a high-fat diet and a single injection of streptozotocin in the low dose.

Materials and methods. Experiments were conducted on 16 outbred male rats. Type 2 diabetes model in experimental animals was achieved by feeding them with high-fat diet (55% of energy from fat) for 28 days followed by a single injection of streptozotocin (35 mg/kg). The serum glucose and insulin concentrations in rats were measured before streptozotocin administration and at the end of the experiment. To estimate insulin resistance, insulin tolerance test and glucose tolerance test were performed. Total protein, albumin, total and direct bilirubin, urea, uric acid, total cholesterol, high-density lipoproteins and low-density lipoproteins, and activity of alanine aminotransferase and aspartate aminotransferase were measured in the blood serum.

Results. A high-fat diet with a single injection of streptozotocin resulted in lipid and protein metabolism disorders and peripheral tissues insulin resistance in experimental animals. Basal insulin levels did not change against the backdrop of high glucose level.

Conclusions. These results indicate that feeding rats with a high-fat diet (55% of calories from fats) and a single administration of streptozotocin at a low dose (35 mg/kg) reproduce general pathological processes of type 2 diabetes. This model can be used to study the pathogenesis of type 2 diabetes as well as to investigate the effect of potential hypoglycemic agents.

Key words: type 2 diabetes, a high-fat diet, streptozotocin, insulin resistance, hyperglycemia.

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Экспериментальная модель сахарного диабета 2-го типа у крыс, вызванная диетой с высоким содержанием жиров и стрептозотоцином в низкой дозе

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РЕЗЮМЕ

Цель исследования – разработать с помощью диеты с высоким содержанием жиров и однократной инъекции стрептозотоцина в низкой дозе патогенетически обоснованную модель сахарного диабета 2-го типа у крыс с выраженной периферической инсулинорезистентностью и относительным дефицитом инсулина.

Материалы и методы. Эксперименты проводили на 16 аутбредных самцах крыс. Сахарный диабет 2-го типа моделировали кормлением экспериментальных животных высокожировой диетой (55% калорий за счет жиров) в течение 28 сут с последующей однократной интраперитонеальной инъекцией стрептозотоцина в дозе 35 мг/кг. Концентрацию глюкозы и инсулина в сыворотке крови крыс измеряли до введения стрептозотоцина и по окончании эксперимента. Для оценки инсулинорезистентности проводили глюкозотолерантный и инсулинотолерантный тесты. В сыворотке крови определяли содержание общего белка, альбуминов, общего и прямого билирубина, мочевины, мочевой кислоты, общего холестерина, холестерина липопротеинов высокой плотности и низкой плотности, активности аланинаминотрансферазы и аспартатаминотрансферазы.

Результаты. Диета с высоким содержанием жиров в сочетании с однократной инъекцией стрептозотоцина приводила у экспериментальных животных к нарушению липидного и белкового обменов и развитию инсулинорезистентности. Уровень базального инсулина не изменялся на фоне выраженной гликемии.

Заключение. Полученные результаты свидетельствуют о том, что при кормлении крыс диетой с высоким содержанием жиров и однократном введении стрептозотоцина в низкой дозе (35 мг/кг) воспроизводятся патологические процессы, характерные для сахарного диабета 2 типа. Созданная модель может использоваться для изучения патогенеза сахарного диабета 2-го типа, а также для исследования действия потенциальных гипогликемических средств.

Ключевые слова: сахарный диабет 2-го типа, высокожировая диета, стрептозотоцин, инсулинорезистентность, гипергликемия.

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

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INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) and obesity has become an epidemic. According to the International Diabetes Federation, more than 422 million people (2017) suffer from diabetes around the world [1]. According to Government Register statistics (2015), about 4.5 million people have type 2 diabetes in the Russian Federation [2]. The key points in the pathogenesis of type 2 diabetes are insulin resistance and pancreatic β-cell dysfunction, which corrupt the regulatory effect of insulin on glucose, protein and lipid metabolism. The progress of diabetes proceeds in several stages. The transition from the state of prediabetes to diabetes in humans progresses during several years [3]. It is necessary to create clinically relevant experimental models of these diseases to develop effective methods for treatment of type 2 diabetes and obesity. It will allow to reproduce the pathogenetic stages of type 2 diabetes formation in a short time.

Genetic models of spontaneous diabetes and models based on pancreatic islet damage by chemical agents are known [4]. To simulate type 2 diabetes, rodents are administered with streptozotocin against the background of a high-fat or high-carbohydrate diet. The antibiotic streptozotocin selectively binds to the pancreatic β-cell marker – the GLUT 2 transporter, then converts into free radicals, causes a detergent effect, and dissociates oxidative phosphorylation that leads to energy deficiency and DNA point mutations with subsequent β-cell necrosis. The severity of β-cell necrosis depends on the route of administration, dose, frequency, and time between streptozotocin injections [5–7]. Variations of these parameters allow to simulate early or late stages of explicit diabetes.

A diet rich in fats and carbohydrates leads to the development of obesity, hyperinsulinemia, insulin resistance and/or glucose intolerance [8, 9]. The ratio of fats, proteins and carbohydrates in the animals' diet and the duration of feeding affect body weight, basal levels of glucose, insulin, triglycerides, cholesterol and fatty acids in plasma. Most often, a high-fat diet with normal amount of carbohydrates is used. Fats of animal (ghee, lard) or vegetable (olive, coconut, soybean) origin are added to the standard diet to obtain energy mainly from fats. Short-term (2 weeks) intake of food enriched with fats, as a rule, causes insulin resistance and/or glucose intolerance. Its longer (more than 4 weeks) intake contributes to an increase in the fat mass, which corresponds to the state of prediabetes [10].

Thus, the combination of streptozotocin injection in a low dose (from 25 mg/kg to 40 mg/kg) and long-term (for 4 weeks to 4 months) feeding of animals with a high-fat diet makes it possible to model pathological characteristics of type 2 diabetes in animals in a short time.

The aim of this study was to develop a pathogenetically reasonable model of type 2 diabetes with marked peripheral insulin resistance and relative insulin deficiency in rats using a high-fat diet and a single injection of streptozotocin in the low dose.

MATERIALS AND METHODS

The experiments were carried out on 16 outbred male rats weighing 300–400 g, obtained from the department of experimental biological models of the E.D. Goldberg Research Institute of Pharmacology and Regenerative Medicine. The animals were kept under standard conditions in a vivarium with natural light and free access to water and food.

The standard ProCorm feed (BioPro, Novosibirsk) for laboratory rats consisted of granules with mineral and vitamin supplements, containing fat 6%, carbohydrates 59%, protein 19%, vitamin–mineral mixture 3%, and water 13%. The energy value of 100 g of feed was 3 660 kcal/kg. It is known that the caloric rate of proteins and carbohydrates is 4 kcal g, fats – 9 kcal/g, therefore, 100 g of this feed provides 236 kcal from carbohydrates, 76 kcal from proteins, 54 kcal from fats and 336 kcal in total. The high-fat diet contained 26 grams of coconut oil, 2 grams of cholesterol and 72 grams of standard food for laboratory animals in 100 g; 55% of the energy was provided by fats [10,13].

The animals were divided into two groups: group 1 – control animals fed with standard laboratory food, group 2 – animals with experimental type 2 diabetes, caused by feeding them with a high-fat diet for 28 days and a single injection of streptozotocin. After a 12-hour fasting, these animals were injected once intraperitoneally with a freshly prepared solution of streptozotocin (35 mg/kg in a 0.1 M citrate buffer at pH 4.5). On the 44th day from the start of the experiment, a glucose tolerance test (GTT) was performed in animals of both groups: 2 g/kg of a 20% glucose solution was injected and after 15, 30, 60 and 120 minutes, fasting glycemia was measured. On the 47th day from the start of the experiment, an insulin tolerance test (ITT) was performed: insulin was administered subcutaneously (NovoRapid Penfil, Novo Nordiks A/C, Denmark) at a dose of 0.75 U/kg. The glucose area under the curve (AUC) was calculated. The body mass of animals was determined during the formation of groups, before the streptozotocin injection and after the end of the experiment. The water and food intake ware measured one day before the end of the experiment.

After the experiment, rats were euthanized by carbon dioxide asphyxia. The content of glucose, total protein, albumin, total and direct bilirubin, urea, uric acid, and the activity of alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) were determined in the serum using the reagent kits and the ARCHITECT C4000 analyzer (USA). The total cholesterol (reagent kits from Randox, UK), and high-density and low-density lipoprotein cholesterol (reagent kits from Chronolab, Spain) were measured spectrophotometrically. The index of atherogenicity (IA) was calculated by the formula: total cholesterol –high density lipoprotein cholesterol / high density lipoprotein cholesterol. The amount of insulin was evaluated by enzyme immunoassay using the ALPCO Diagnostics (USA) ELISA kit. The HOMA-IR index (insulin content, pmol/L*glucose, mmol/L)/155) [https://www.dtu.ox.ac.uk/homacalculator/download.php] and the constant glucose utilization rate based on ITT (KITT) were calculated to characterize the insulin resistance [12].

The results were processed using the methods of one-way analysis of variance using the SPSS Statistics 12.0 package. Quantitative indicators were presented as the median, 25th and 75th percentiles. When comparing two independent samples, the Mann – Whitney test was used. The significance of differences was achieved at $p < 0.05$.

RESULTS AND DISCUSSION

The body weight of control animals steadily increased throughout the experiment (Table 1). The body weight of animals in the group with the T2DM model (receiving a high-fat diet) increased more significantly before the steptozotocin injection, the serum insulin concentration increased by 20%, the HOMA-IR index, which characterizes the development of glucose tolerance, became 23% higher. Compensatory increase in insulin secretion prevented the development of hyperglycemia in the serum of experimental animals in the second group (Table 1). Weight gain, hyperinsulinemia, insulin resistance and the absence of hyperglycemia confirm the development of prediabetes in animals with the T2DM model. Partial loss of the β-cell functional mass is necessary for the transition from prediabetes to explicit type 2 diabetes, therefore, animals were injected once intraperitoneally with streptozotocin on the 29th day of the experiment (35 mg/kg).

On the 50th day of the experiment (21 days after the administration of streptozotocin), the body weight of the animals decreased by 10.7% compared to the weight of control rats. Such a decrease in body weight is probably associated with the transition of energy products from the carbohydrate oxidation to the oxidation of fats. The daily water consumption by animals of the second group increased by 3.9 times. The amount of feed intake in both groups did not differ (Table 1), but the energy intensity of food was significantly higher for animals in the second group.

Table 1

The effect of a high-fat diet (55% calories from fats) and streptozotocin (single injection, 35 mg/kg) on body weight, food and water consumption, glucose and insulin serum levels in rats, *Me* [Q_1; Q_3]

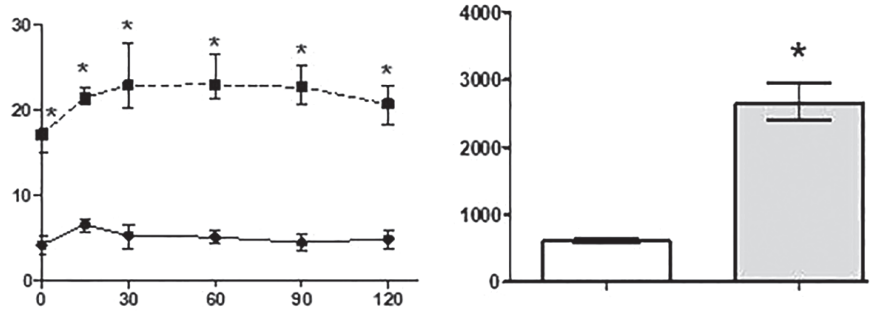
Parameter		Experimental groups	
		Control $n = 8$	High-fat diet + streptozotocin $n = 8$
Glucose, mmol/l		5.2 [4.6; 5.6]	5.6 [5.2; 5.9]
Insulin, pg/ml		229.1 [212.7; 234.0]	280.1 [260.5; 284.3]*
HOMA-IR		1.3 [1.1; 1.4]	1.7 [1.6; 1.8]*
Weight, g	0th day	311.0 [305.0; 320.0]	319.0 [310.0; 323.0]
	29th day	424.0 [398.0; 439.0]	459.0 [426.0; 481.0]*
	50th day	465 [446; 475]	420 [380; 447]*
Food intake, g/day		31.2 [30.0; 36.0]	36.7 [31.9; 39.1]
Water intake, ml/day		49.0 [39.4; 54.9]	189.5 [147.9; 205.4]*

* $p < 0.05$ comparing the experimental group with the controls.

15 days after the streptozotocin administration, GTT revealed significant impaired glucose sensitivity compared to the control group. The initial blood glucose level was significantly elevated after nightly fasting and at all time intervals after glucose administration (Fig. 1) in rats with the experimental T2DM model. In the T2DM model, the area under the curve increased by 4.4 times compared with the area in the control.

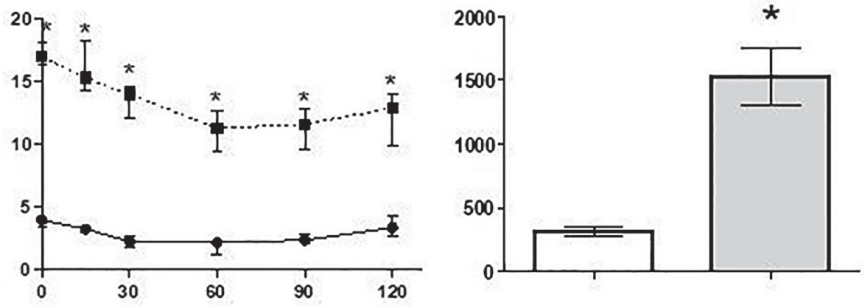
During the ITT, which characterizes the sensitivity of tissues to exogenous insulin, the concentration of glucose in the blood of control animals decreased as much as possible (2.3 [1.9; 2.8] mmol/L) at 30 minutes after the insulin administration (0.75 U/kg). In the blood of animals with the model of type 2 diabetes, the concentration of glucose decreased as much as possible after 60 minutes (11.3 [9.4; 12.7] mmol/l) (Fig. 2). It indicates slow utilization of glucose by peripheral tissues due to the development of insulin resistance. According to ITT data, the area under the curve in animals with the experimental T2DM model became 4.8 times larger than in the control group (Fig. 2). K_{ITT}, calculated on the basis of ITT, in animals of the control group was 2.7 [1.9; 3.1] % glucose/min. In the group of animals with the experimental T2DM model, the glucose utilization rate decreased by 53% (1.4 [1.0; 1.7] % glucose/min) (Fig. 3).

The pathogenesis of type 2 diabetes is characterized by a combination of tissue resistance to insulin and insufficient pancreatic β-cell function [13]. Violation of carbohydrate homeostasis in animals with experimental type 2 diabetes is confirmed by a three-fold increase in the serum glucose concentration (Table 2). The insulin



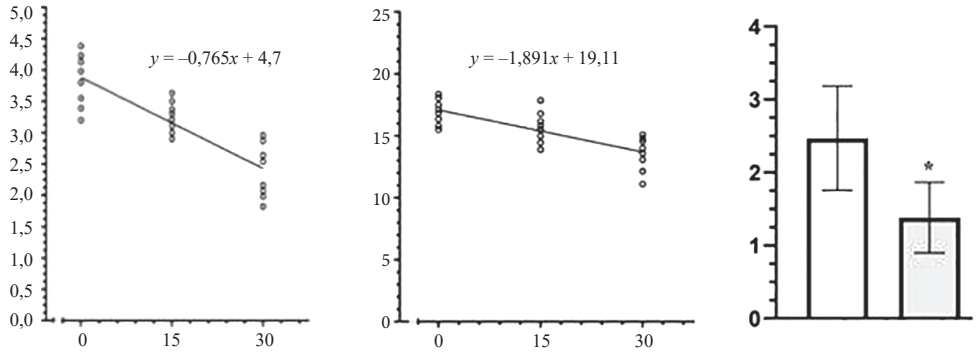
a *b*

Fig. 1. The glucose tolerance test results in male Wistar rats fed with high-fat diet (55% calories from fats) and administered with a single intraperitoneal injection of streptozotocin (35 mg/kg) (day 15 after injection): *a* – the blood glucose concentration dynamics in the control (solid line, $n = 8$) and experimental (dashed line, $n = 8$) rats after intraperitoneal administration of glucose (2 g/kg). The abscissa axis represents the time after intraperitoneal glucose administration, min; the ordinate axis represents the blood glucose concentration, mmol/l; *b* – the area under the curve "glucose concentration – time" in the control (light column) and type 2 diabetes model (dark bar) groups, min × mmol/l. * the significance of differences compared with the control group, $p < 0.05$



a *b*

Fig. 2. The insulin tolerance test results in male Wistar rats fed with high-fat diet (55% calories from fats) and administered with a single intraperitoneal injection of streptozotocin (35 mg/kg) (day 18 after injection): *a* – the blood glucose concentration dynamics in the control (solid line, $n = 8$) and experimental (dashed line, $n = 8$) rats after subcutaneous administration of insulin (0.75 U/kg). The abscissa axis represents the time after subcutaneous administration of insulin, min; the ordinate axis represents the blood glucose concentration, mmol/l; *b* – the area under the curve "glucose concentration – time" in the control (light column) and type 2 diabetes model (dark bar) groups, min × mmol/l. * the significance of differences compared with the control group, $p < 0.05$



a *b* *c*

Fig. 3. The insulin tolerance test results in male Wistar rats fed with high-fat diet (55% calories from fats) and administered with a single intraperitoneal injection of streptozotocin (35 mg/kg) (day 18 after injection): a and *b* – blood glucose concentration dynamics in the control (*a,* $n = 8$) and type 2 diabetes model (*b*, $n = 8$) groups after subcutaneous administration of insulin (0.75 U/kg). The abscissa axis represents the time after subcutaneous administration of insulin, min; the ordinate axis represents the blood glucose concentration, mmol/l; c – the glucose rate utilization constant (K_{ITT}) during the insulin tolerance test in the control (light column) and type 2 diabetes model (dark column) rats, $\%_{glucose}$/min. * significance of differences compared with the control group, $p < 0.05$

content in the serum of control animals and animals with the T2DM model did not differ, but the HOMA-IR was 2.8 times higher in animals with the experimental T2DM model (Table 2). Streptozotocin causes pancreatic β-cell necrosis, while a compensatory increase in their mass and insulin secretion is observed [13]. A high level of glucose in the serum of animals with the experimental T2DM model and the same insulin level as in the control group indicate the impossibility of insulin resistance compensation by increased insulin secretion. Such disturbances are characteristic of the late stage of T2DM. Gluconeogenesis and glycogenolysis intensify, and glycogen synthesis in the liver and skeletal muscles decreases in animals with the T2DM model [1].

Lipid and protein metabolism also disrupts during type 2 diabetes progression. In the serum of animals with experimental type 2 diabetes, the content of total cholesterol increased significantly (by 7.5 times), the amount of low-density lipoprotein cholesterol elevated, the atherogenic index rose, and the high-density lipoprotein cholesterol was not changed. Impaired lipid metabolism was accompanied by the development of dyslipidemia, one of the most common causes of cardiovascular complications of diabetes (Table 2) [11]. In animals with the T2DM model, the content of circulating free fatty acids and triglycerides increased by 2 times due to stimulation of lipolysis in adipose tissue caused by insulin resistance. Free fatty acids accumulated in the liver triglycerides and ectopic tissues, which further exacerbated insulin resistance. The urea and uric acid content became 2.8 and 1.6 times higher in animals with the T2DM model than in the control. These metabolic disorders are explained by the activation of protein catabolism, mainly in the muscles and liver. The symptoms of liver dysfunction appeared in animals with the T2DM model: the content of albumin decreased in the serum, the activity of the hepatic enzyme ALT increased, while the activity of AST and the concentration of bilirubin did not differ from the control group. (Table 2).

Table 2

The effect of a high-fat diet (55% calories from fats) and streptozotocin (single injection, 35 mg/kg) on biochemical blood parameters in rats, *Me* [Q_1; Q_3]

Parameter	Control (n = 8)	T2DM model (n = 8)
Alanine aminotransferase, U/L	53.0 [49.0; 57.5]	97.0 [93.,0; 109.0]*
Aspartate aminotransferase, U/L	113.0 [108.0; 134.5]	134.0 [121.5; 146.0]
Total bilirubin, mmol/l	2.6 [2.4; 2.9]	3.2 [2.5; 4.2]
Direct bilirubin, mmol/l	1.6 [1.4; 1.7]	1.8 [1.5; 3.1]
Total protein, g/l	76.0 [72.0; 79.0]	72.5 [71.0; 75.5]
Albumin, g/l	36.5 [35.0; 37.0]	32.0 [31.5; 33.0]*
Urea, mmol/l	5.5 [5.0; 5.7]	15.8 [14.5; 17.8]*
Uric acid, mmol/l	165.0 [155; 182.5]	266.0 [236.0; 282.0] *
Total cholesterol, mmol/l	1.8 [1.6; 2.6]	13.6 [8.3; 17.3]*
Low-density lipoprotein cholesterol, mmol/l	0.6 [0.4; 0.7]	5.6 [3.8; 7.6]*
High-density lipoprotein cholesterol, mmol/l	1.0 [0.8; 1.2]	1.0 [0.8; 1.2]
Index of atherogenicity	1.0 [0.6; 1.5]	14.0 [8.9; 16.6]*
Free fatty acids, mM	0.7 [0.6; 0.8]	1.4 [1.3; 1.,8]*
Triacylglycerols, mmol/l	1.2 [0.7; 1.4]	5.1 [2.9; 7.0]*
Glucose, mmol/l	5.1 [4.8; 5.3]	16.9 [15.9; 17.6]*
Insulin, pg/ml	328.8 [229.1; 520.4]	355.9 [279.1; 521.2]
HOMA-IR	1.9 [1.2; 4.5]	5.4 [3.9; 8.4]*

* $p < 0.05$ comparing the experimental group with the control.

Thus, in this model it is possible to trace the main manifestations of carbohydrate, lipid, and protein metabolism disruptions, which are characteristic of the type 2 diabetes pathogenesis.

CONCLUSION

The obtained results indicate that rats fed with a high-fat diet (55% calories from fats) and administered with a single injection of streptozotocin in the low dose (35 mg/kg) reproduce the pathological processes of T2DM. The level of basal insulin does not change, but hyperglycemia is pronounced. This indicates the emergence of insulin resistance in peripheral tissues. Metabolic changes correlate with the results obtained during ITT and GTT and a higher HOMA-IR index. The created model can be used to study the pathogenesis of type 2 diabetes and to investigate the effects of potential hypoglycemic agents.

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Kaydash O.A. – conception and design, review of publications on the topic, analysis of the obtained data, drafting of the manuscript. Ivanov V.V. – final approval of the manuscript for publication. Vengerovsky A.I. – critical revision of the manuscript for important intellectual content, approval of the manuscript for publication. Buyko E.E. – analysis of the obtained data, drafting of the manuscript. Shchepetkin I.A. – critical revision of the manuscript for important intellectual content, approval of the manuscript for publication.

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Human neutrophil antigen allele frequencies and assessment of HNA alloimmunization risk in donors and hematological patients

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ABSTRACT

Human neutrophil antigens (HNAs) are localized on glycoproteins which are positioned on the surface membrane of human neutrophils. Alloantibodies against HNA are implicated in a number of clinical conditions, including immune-mediated neutropenia and transfusion reactions. Genotyping for HNA systems is important in the diagnosis of disorders involving alloimmunization to HNA.

Aim. To assess the risk of HNA alloimmunization in donors and patients with hematological diseases in St. Petersburg based on the study of HNA allele and genotype frequencies.

Materials and methods. DNA samples of 303 blood donors and 302 hematological patients were obtained and typed for HNA-1, -3, -4, -5. Polymerase chain reactions with homemade sequence-specific primers were used for typing. Genomic DNA was isolated from whole blood by a multistage purification method using the CTAB reagent. The results were detected in real time using the EVAGreen intercalating dye. Pearson's chi-squared test was used to compare the HNA genotype frequencies in donors, patients with hematological diseases and in other populations.

Results. In the study, the frequency of HNA-1bd allele was 0.584–0.588, of HNA-1a – 0.376–0.384, of HNA-1bc – 0.032–0.036. HNA-1bc allele was represented in the genotypes HNA-1a/bc/bd (0.023–0.036), HNA-1a/bc (0.020–0.043) and HNA-1bc/bd (0.007–0.010). The genotypes HNA-1bc/bc and HNA-1null were not identified. Allele "a" of HNA-3, -4, -5 systems was found in the majority of studied individuals (0.795–0.804; 0.887–0.898; 0.699–0.708). The highest calculated risk of HNA alloimmunization was noted in the absence of HNA-5b, HNA-1a, HNA-3b, and HNA-4b alleles in the genotype and was 0.250, 0.233, 0.231, and 0.163, respectively.

Conclusions. Our data are consistent with the results of studies on the HNA allele and genotype frequencies in populations of Europeans and are significantly different from those of East and Southeast Asia, Africa and South America. The frequencies of HNA-1, -3, -4, -5 alleles and genotypes among donors in St. Petersburg and patients with hematological diseases did not have statistically significant differences. It was shown that the highest calculated risk of alloimmunization was observed in the absence of HNA-5b, HNA-1a, HNA-3b, and HNA-4b alleles in the genotype. These data are consistent with the results of similar studies on populations of white Europeans conducted by other authors.

Key words: donor, neutrophil antigens, genotyping.

Conflicts 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 participants signed an informed consent to participate in the study and have blood samples collected. The study was approved by the local Ethics Committee of Russian Research Institute of Hematology and Transfusiology (Protocol No. 56 dated December 26, 2018).

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Частота встречаемости антигенов нейтрофилов человека и риск аллоиммунизации у доноров и больных гематологическими заболеваниями

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РЕЗЮМЕ

Актуальность. Антигены нейтрофилов человека (Human neutrophil antigens, HNA) локализованы на гликопротеинах, расположенных на поверхностной мембране нейтрофилов. Иммунизация к HNA во время беременности или вследствие трансфузий компонентов крови может привести к выработке аллоантител. Одним из факторов развития аллоиммунизации является частота встречаемости HNA. В связи с этим представляется важным изучить особенности распределения аллелей и генотипов HNA у доноров и больных гематологическими заболеваниями г. Санкт-Петербурга для прогнозирования риска аллоиммунизации.

Цель. Оценить риск HNA аллоиммунизации у доноров и больных гематологическими заболеваниями г. Санкт-Петербурга на основании изучения частот встречаемости аллелей и генотипов HNA.

Материалы и методы. Материалом исследования служили образцы периферической крови 303 доноров г. Санкт-Петербурга и 302 больных гематологическими заболеваниями, получавших терапию в Российском научно-исследовательском институте гематологии и трансфузиологии. Геномная ДНК была выделена из цельной крови методом многоступенчатой очистки с использованием реактива *цетилтриметиламмония бромида.* Типирование HNA проводили методом аллель-специфичной полимеразной цепной реакции с использованием разработанных олигонуклеотидных праймеров. Сравнения частот встречаемости генотипов HNA у доноров, больных гематологическими заболеваниями, и представителей других популяций проводили с помощью критерия согласия Пирсона χ^2.

Результаты. Частота встречаемости аллеля HNA-1bd составила 0,584–0,588, а HNA-1a – 0,376–0,384. Частота встречаемости аллеля HNA-1bc составила 0,032–0,036, и данный аллель был представлен в генотипах HNA-1a/bc/bd (0,023–0,036)00, HNA-1a/bc (0,020–0,043) и HNA-1bc/bd (0,007–0,010). Генотипы HNA-1bc/bc и HNA-1null выявлены не были. Аллель «а» систем HNA-3, -4, -5 встречался у большинства исследуемых в каждой группе (0,795–0,804; 0,887–0,898; 0,699–0,708 соответственно). На основании полученных частот встречаемости аллелей и генотипов рассчитали вероятность аллоиммунизации к HNA. Наибольшая величина расчетного риска аллоиммунизации при трансфузиях компонентов крови отмечена при отсутствии в генотипе аллелей HNA-5b, HNA-1a, HNA-3b, HNA-4b и составляет 0,250; 0,233; 0,231 и 0,163 соответственно, что подтверждает результаты аналогичных исследований.

Заключение. Статистически значимых различий в частоте встречаемости аллелей и генотипов HNA-1, -3, -4, -5 у доноров г. Санкт-Петербурга и больных гематологическими заболеваниями не установлено. Наибольшая величина расчетного риска аллоиммунизации при трансфузиях компонентов крови, полученная на основании частот встречаемости аллелей и генотипов, отмечена при отсутствии в генотипе аллелей HNA-5b, HNA-1a, HNA-3b, HNA-4b. Полученные данные согласуются с результатами исследований распределения аллелей и генотипов систем HNA в популяции европейцев и значимо отличаются от популяций Восточной и Юго-Восточной Азии, Африки и Южной Америки. Предложенный метод типирования HNA может быть использован для создания клеточной панели, типированной по антигенам нейтрофилов, с целью определения специфичности аллоантител у доноров, и для диагностики аллоиммунных конфликтов в педиатрии, трансфузиологии и трансплантологии.

Ключевые слова: антигены нейтрофилов, генотипирование, аллель-специфичная полимеразная цепная реакция.

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

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

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании и согласие на забор крови. Протокол исследования одобрен локальным этическим комитетом ФГБУ РосНИИГТ ФМБА России (протокол № 56 от 26.12.2018).

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INTRODUCTION

Human neutrophil antigens (HNAs) are localized on glycoproteins, which are positioned on the surface membrane of neutrophils. Immunization against HNA during pregnancy or as a result of blood component transfusion can lead to the production of alloantibodies to neutrophil antigens. HNA antibodies can cause development of such clinical conditions as neonatal alloimmune neutropenia (NAN), autoimmune neutropenia (AIN), transfusion-related acute lung injury (TRALI), febrile non-hemolytic transfusion reactions, immune neutropenia after bone marrow transplantation, and drug-induced immune neutropenia [12]. In addition, according to the literature, HNA polymorphism is a risk factor not only for the above mentioned conditions, but also for other diseases, including bacterial infections (periodontitis), chronic inflammatory diseases (vasculitis, systemic lupus erythematosus, rheumatoid arthritis), and susceptibility to malaria [3]. There is no information on the distribution of HNA among donors in the Russian Federation.

To date, 5 HNA systems (HNA-1, -2, -3, -4, -5) have been described [1].

The HNA-1 system includes HNA-1a, HNA-1b, HNA-1c, and HNA-1d antigens located on the Fc-γ receptor IIIb (FcγRIIIb, CD16b). The expression of HNA-1b is always accompanied by the expression of HNA-1d or HNA-1c. FcγRIIIb is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed on the neutrophil surface and encoded by the *FCGRIIIB* gene [4].

The HNA-2a antigen is located on the GPI-anchored protein CD177 encoded by the *CD177* gene [5]. HNA-2a is found in most individuals; the absence of HNA-2a is determined by a defect in the transcription of the CD177 gene [6].

HNA-3a and HNA-3b are located on the choline transporter-like protein 2 (CTL 2) encoded by the *SLC44A2* gene [7].

The antigens of the HNA-4 and HNA-5 systems are localized on the αM (CD11b) and αL (CD11a) integrin subunits and encoded by the *ITGAM* and *ITGAL* genes, respectively [8].

The presence of each allele of the HNA-1 system is determined by a combination of 6 single nucleotide polymorphisms (SNPs) in the *FCGRIIIB* gene, located close to one other. The HNA-3, -4, -5 systems include two alleles each, the differences between which are determined by replacement of one nucleotide in the DNA sequence of the corresponding genes [1].

The frequency of HNA is one of the factors of immune response development [9]. In this regard, it seems important to study the distribution of HNA alleles and genotypes in the population. Serological and molecular genetic methods are used to study the frequency of HNA. However, serological typing of neutrophil antigens can be complicated due to the absence of some typing reagents, high cost and short lifetime of neutrophils. Polymerase chain reaction (PCR)-based methods are the most optimal for HNA-1, -3, -4, -5 typing [10], but are often not used due to a lack of regulatory documents and test systems for HNA typing from domestic manufacturers.

The aim of the study was to assess the risk of HNA alloimmunization in donors and patients with hematological diseases in St.-Petersburg based on the study of HNA genotype and allele frequencies.

MATERIALS AND METHODS

Peripheral blood samples were obtained from 303 donors from St.-Petersburg and 302 patients with hematological diseases, who received therapy at Russian Research Institute of Hematology and Transfusiology.

HNA typing was performed by allele-specific PCR (AS-PCR) using homemade oligonucleotide primers. Genomic DNA was isolated from whole blood by the multistage purification method using etyltrimethylammonium–bromide (CTAB) reagent. The results were detected in real time using the EVAGreen intercalating dye.

The results were statistically processed using the Statistica 7 software. Comparison of allele frequencies and HNA genotypes in donors, patients with hematological diseases, and in other populations, as well as check of the correspondence of the observed distributions to the Hardy – Weinberg equilibrium were performed using the Pearson's chi-squared test (χ^2). The differences with $p < 0.05$ were considered statistically significant. The critical χ2 value for alleles of

the HNA-3, -4, -5 systems with *p*-value of 0.05 was 3.84. The critical χ2 value for the alleles of the HNA-1 system with *p*-value of 0.05 was 5.991.

The following formulas were used to estimate the probability of HNA alloimmunization:

– the probability of alloimmunization to the allele "a" = (aa + ab) × bb,

– the probability of alloimmunization to the allele "b" = (bb + ab) × aa,

– where aa, ab, bb are the frequencies of the corresponding genotypes.

RESULTS

Oligonucleotide primers for HNA typing by AS-PCR method were designed using Primer 3.0 and Primer-BLAST. The sequences of oligonucleotide primers for typing antigens of the HNA-1, -3, -4, -5 systems are shown in Table 1.

Table 1

DNA sequences of oligonucleotide primers for HNA-1,-3,-4,-5 genotyping

Antigen	Gene	Forward primer (5'– 3')	Reverse primer (5'– 3')
HNA-1a	*FCGR3B*	CCTCAATGGTACAG**G**GTGCT**C**	GCCTGGCTTGAGATGAGG**T**T
HNA-1b/c	*FCGR3B*	CCTCAATGGTACAG**C**GTGCT**T**	CACTGTCGTTGACTGTGGCA**T**
HNA-1b/d	*FCGR3B*	CCTCAATGGTACAG**C**GTGCT**T**	ACTGTCGTTGACTGTGGCA**G**
HNA-3a	*SLC44A2*	CTACCTCACGTACCTGAATGCT	GCAGGGCAGTCACCATCT**C**
HNA-3b	*SLC44A2*	CTACCTCACGTACCTGAATGCT	GCAGGGCAGTCACCATCT**T**
HNA-4a	*ITGAM*	CTCATGCGAGCCCATCC**G**	ACAAGGAGGTCTGACGGTGA
HNA-4b	*ITGAM*	CTCATGCGAGCCCATCC**A**	ACAAGGAGGTCTGACGGTGA
HNA-5a	*ITGAL*	ATCATCCCCCACAGATCCA**G**	AGCTGGACCCAGTAAGCATC
HNA-5b	*ITGAL*	ATCATCCCCCACAGATCCA**C**	AGCTGGACCCAGTAAGCATC

Note. nucleotides complementary to SNPs that determine the antigen presence are shown in bold.

To analyze the specificity of the primers, DNA samples from 20 donors were used the sequences of the HNA-1, -3, -4, -5 alleles in which were determined by sequencing. The correspondence of the results of sequencing and AS-PCR with the real-time results and agarose gel electrophoresis was 100%. No nonspecific PCR products were identified by gel electrophoresis.

The real-time PCR conditions for the HNA-1, -3, -4, -5 allele typing were the same. For the analysis we used a 2.5x PCR reaction mix in the presence of EVA-Green (SINTOL, Moscow), which includes a 2.5x PCR buffer B (6.25 mmol $MgCL_2$, KCl, TrisHCl (pH 8.8)), SynTaq DNA polymerase, deoxynucleoside triphosphates, glycerol, and Tween 20. For PCR, a mixture containing 50–100 ng of genomic DNA, 1x PCR reaction mix, and 0.2 μmol of forward and reverse primers was added to each tube. The final volume of the mixture was adjusted to 25 μl with double-distilled water. The following protocol was used for PCR: 95° for 5 min, then 33 cycles: 95° – 20 sec, 68° – 30 sec.

HNA-1, -3, -4, -5 allele and genotype frequencies in donors of St.-Petersburg and patients with hematological diseases are shown in Table 2. The deviation of the observed genotype distribution from the one expected in patients with hematological diseases and donors was not statistically significant.

Table 2

Comparison of frequencies of genotypes and alleles of HNA systems in donors and patients with hematological diseases

System	Genotype	Patients, *n* = 302	Donors, *n* = 303	χ^2	Allele	Patients	Donors
HNA-1	a/a	0.142	0.142	$\chi^2 = 0.172$, $p = 0.918$	a	0.376	0.384
	a/bc/bd	0.023	0.036		bd	0.588	0.584
	a/bc	0.043	0.020		bc	0.036	0.032
	a/bd	0.411	0.442		–	–	–
	bc/bd	0.007	0.010		–	–	–
	bd/bd	0.374	0.350		–	–	–
HNA-3	a/a	0.623	0.650	$\chi^2 = 0.150$, $p = 0.699$	a	0.795	0.804
	a/b	0.343	0.307		b	0.205	0.196
	b/b	0.033	0.043		–	–	–

Table 2 (continued)

HNA-4	a/a	0.788	0.802	$\chi^2 = 0.321$, $p = 0.571$	a	0.887	0.898
	a/b	0.199	0.191		b	0.113	0.102
	b/b	0.013	0.007		–	–	–
HNA-5	a/a	0.500	0.488	$\chi^2 = 0.124$, $p = 0.725$	a	0.699	0.708
	a/b	0.397	0.439		b	0.301	0.292
	b/b	0.103	0.073		–	–	–

As can be seen from Table 2, HNA-1, -3, -4, -5 genotype frequencies in patients with hematological diseases who received therapy at Russian Research Institute of Hematology and Transfusiology had no statistically significant differences from those in donors of St. Petersburg. In the examined groups, the frequency of HNA-1bd allele was higher (0.584–0.588) than HNA-1a (0.376–0.384). The frequency of HNA-1bc was 0.032–0.036, and this allele was represented in the genotypes HNA-1a/bc/bd (0.023–0.036), HNA-1a/bc (0.020–0.043), and HNA-1bc/bd (0.007–0.010). The genotypes HNA-1bc/bc and HNA-1null were not identified.

The allele "a" of the HNA-3, -4, -5 systems was found in the majority of individuals in each group (0.795–0.804; 0.887–0.898; 0.699–0.708, respectively). The prevalence of HNA-5a was 0.699–0.708.

Since HNA-1, -3, -4, -5 genotype frequencies in patients with hematological diseases and donors of St. Petersburg did not have statistically significant differences, an assessment of the possible risk of HNA alloimmunization was calculated based on the data on the HNA allele and genotype frequencies in the group that included both donors and patients with hematological diseases (Table 3).

Table 3

Assessment of the possible risk of alloimmunization against HNA-1, 3-5 during transfusions of blood components

Risk of HNA alloimmunization								
HNA 1a	HNA 1bd	HNA 1bc	HNA 3a	HNA 3b	HNA 4a	HNA 4b	HNA 5a	HNA 5b
0.233	0.143	0.064	0.037	0.231	0.01	0.163	0.080	0.250

As can be seen from the presented data, the highest calculated risk of alloimmunization during blood component transfusion was observed in the absence of HNA-5b, HNA-1a, HNA-3b, and HNA-4b alleles in the genotype and was 0.250, 0.233, 0.231, and 0.163, respectively.

DISCUSSION

The data obtained are consistent with the results of studies on the distribution of HNA alleles and genotypes in European populations [9] and significantly differ from other populations. Thus, in the populations of South – East Asia, China, and Japan, the HNA-1a allele frequency was significantly higher and was 0.696, 0.667, and 0.623, respectively ($p < 0.001$) [11]. The frequency of HNA-3a was 0.795 – 0.804, which was significantly higher than that in the Japanese population (0.654) [12], and significantly lower than in the populations of Zambia (0.974) and Brazil (1.0) ($p < 0.001$) [13, 14]. The frequency of HNA-4a was 0.887–0.898, and this was significantly lower than in the populations of China and Brazil – 0.995 and 1.0, respectively ($p < 0.001$) [15, 14]. The obtained frequency of HNA-5a was significantly higher than in the Zambian population (0.500) [13], but significantly lower than in the populations of China (0.852), Japan (0.840), and Brazil (0.855) ($p < 0.001$) [15, 12, 14]. The probability of HNA alloimmunization was calculated based on the obtained allele and genotype frequencies. The highest calculated risk of alloimmunization during blood component transfusion was observed in the absence of HNA-5b, HNA-1a, HNA-3b, and HNA-4b alleles in the genotype and was 0.250, 0.233, 0.231, and 0.163, respectively, which confirms the results of similar studies conducted by other authors in the population of white Europeans [10]. The obtained data may be useful for predicting clinical conditions associated with alloimmunization. However, the probability of immune response development is highly dependent on the immunogenicity of the antigen and other factors, which may explain the discrepancy between the calculated risk of alloimmunization and the existing data on the specificity of detected HNA alloantibodies. Thus, for example, at low calculated risk of alloimmunization to HNA-3a (0.37), such antibodies lead to the development of TRALI. It is known that anti-HNA-3a alloantibodies contained in donor plasma are one of the reasons for the development of severe TRALI with fatal outcome [12]. Despite high calculated risk of HNA-3b alloimmunization, rare cases of NAN caused by anti-HNA-3b alloantibodies have been described, and there are no data on the cases of TRALI caused by such antibodies in the literature.

The most common causes of NAN development in the population of white Europeans are alloantibodies to HNA-1a, -1b, -1c and -2a [16]. However, there are cases of NAN caused by alloantibodies to HNA-1d, -3a, -3b, -4a , -4b, -5a that are described in the literature [17, 18].

In clinical practice, transfusions of granulocyte concentrate are carried out for patients with a significant decrease in the absolute number of granulocytes in the blood in the presence of infection, uncontrolled antibiotic therapy, and in sepsis of newborns. In case of transfusion of HNA incompatible blood component, the patient has an increased risk of alloimmunization and, as a result, there is a lack of clinical effect of transfusion [19]. Individual selection of HNA and HLA-compatible blood components is required for such patients. Building of a HNA-typed donor base will help prevent alloimmunization. The development of a HNA-typed cell panel will help solve the problem of diagnosing alloimmune conflicts in pediatrics, transfusiology and transplantology.

CONCLUSION

The conducted research allowed to study the distribution patterns of HNA alleles and genotypes in donors of St.-Petersburg and patients with hematological diseases and to assess the possible risk of HNA alloimmunization. No statistically significant differences in the frequencies of HNA-1, -3, -4, -5 alleles and genotypes were found in donors of St.-Petersburg and patients with hematological diseases. The highest estimated risk of alloimmunization during transfusion of blood components was observed in the absence of HNA-5b, HNA-1a, HNA-3b, an HNA-4b alleles in the genotype, which confirms the results of similar studies conducted by other authors in th population of white Europeans. The data obtained may be useful for predicting clinical conditions associated with alloimmunization. However, the immunization process after transfusion and / or during pregnancy is associated not only with antigenic incompatibility, but also depends on the immunogenicity of the antigen, genetic, epigenetic and environmental factors, which may explain the discrepancy between the calculated risk of alloimmunization and the existing data on the specificity of detected HNA alloantibodies.

The presented data may be useful for prevention of alloimmunization, as well as for population studies. The proposed HNA typing method can be used to develop a HNA-typed cell panel in order to determine the specificity of alloantibodies in donors and to diagnose alloimmune conflicts in pediatrics, transfusiology and transplantology.

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Endothelial monolayer disruption in the bioprosthetic heart valve as a trigger of primary tissue failure

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ABSTRACT

Aim. To study the surface and cellular composition of non-calcified bioprosthetic heart valve (BHV) leaflets with varying degrees of structural deterioration to determine the possible mechanisms of primary tissue failure development.

Materials and methods. An examination of six bioprosthetic heart valves (KemCor and PeriCor) extracted from mitral position due to the structural valve deterioration was performed. The structure of BHV leaflets was studied by hematoxylin – eosin staining and immunohistochemistry assay (with the following indicators – CD3, T lymphocytes; CD20, B lymphocytes; CD31, mature endothelial cells; CD34, endothelial progenitor cells; CD68, monocytes/macrophages; vimentin, mesenchymal cells; α-smooth muscle actin, vascular smooth muscle cells).

Results. The degree of disruption of BHV leaflets in primary tissue failure differed significantly: relatively intact samples with the intact endothelial monolayer, areas with impairment of the surface layers (minimal and moderate damage) and areas with the spread of destruction into the extracellular matrix of the leaflet (expressed degeneration) were determined. Endothelial cells (monolayer with preserved or impaired integrity), macrophages, smooth muscle cells and other mesenchymal lineage cells were identified in BHV. T- and B-lymphocytes were not detected in the BHV leaflets.

Conclusions. A characteristic feature of structurally deteriorated BHVs is impairment of endothelial monolayer integrity in areas of degraded extracellular matrix. In contrast to other types of bioprosthetic dysfunctions, structural valve deterioration was characterized by the absence of lymphocyte infiltration. Therefore, we suppose that endothelial mololayer injury is a trigger of structural BHV deterioration.

Key words: bioprosthetic heart valves, structural valve deterioration, extracellular matrix.

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Нарушение целостности эндотелиального монослоя биопротезов клапанов сердца как триггер развития первичной тканевой несостоятельности

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РЕЗЮМЕ

Цель – морфологическое исследование поверхности и клеточного состава створок некальцинированных биопротезов клапанов сердца (БКС) с различной степенью их повреждения для определения возможных механизмов развития первичной тканевой несостоятельности (ПТН).

Материалы и методы. Исследовано шесть ксеноаортальных клапанов «КемКор» и «ПериКор», извлеченных из митральной позиции по причине развития ПТН. Структуру створок БКС и особенности ее изменения изучали гистологическим (окраска гематоксилин-эозином) и иммуногистохимическим методами. Иммуногистохимическое исследование БКС включало идентификацию маркеров: CD3 (Т-лимфоциты), CD20 (В-лимфоциты), CD31 и CD34 (эндотелиальные клетки), CD68 (моноциты/макрофаги), виментин (клетки мезенхимального ряда), α-гладкомышечный актин (гладкомышечные клетки).

Результаты. Степень нарушения структуры створок БКС при ПТН существенно различалась: определялись относительно сохранные образцы с интактным эндотелиальным монослоем на поверхности створки, образцы с минимальным или умеренным нарушением структуры эндотелиального слоя и образцы с выраженной деструкцией эндотелиального слоя створки БКС. В составе БКС были идентифицированы эндотелиальные клетки (монослой с сохраненной или нарушенной целостностью), макрофаги, гладкие миоциты и прочие клетки мезенхимального происхождения. Следует отметить, что нами не обнаружено Т- и В-лимфоцитов в створках БКС.

Заключение. Характерным признаком структуры БКС, эксплантированных по причине ПТН, является нарушение целостности эндотелиального монослоя в участках дезинтеграции экстрацеллюлярного матрикса. Кроме того, в сравнении с другими типами протезных дисфункций ПТН отличается отсутствием

лимфоцитарной инфильтрации. На основании полученных данных можно сделать вывод о триггерной роли дезинтеграции эндотелиального монослоя в развитии ПТН.

Ключевые слова: биопротезы клапанов сердца, первичная тканевая несостоятельность, экстрацеллюлярный матрикс.

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

Источник финансирования. Работа выполнена при поддержке комплексной программы фундаментальных научных исследований СО РАН в рамках фундаментальной темы Научно-исследовательского института комплексных проблем сердечно-сосудистых заболеваний № 0546-2015-0011 «Патогенетическое обоснование разработки имплантатов для сердечно-сосудистой хирургии на основе биосовместимых материалов, с реализацией пациент-ориентированного подхода с использованием математического моделирования, тканевой инженерии и геномных предикторов».

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Протокол исследования одобрен локальным этическим комитетом ФГБНУ «Научно-исследовательский институт комплексных проблем сердечно-сосудистых заболеваний» (протокол № 8 от 14.05.2019).

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INTRODUCTION

A generally recognized disadvantage of bioprosthetic heart valves (BHV) is limited duration of their functioning explained by the development of structural insufficiency of the implanted xenomaterial – primary tissue failure (PTF), under the influence of various factors associated with the characteristics of the implants and / or recipient organism [1, 2]. The most common types of BHV dysfunctions requiring surgery to replace the prosthesis are tissue calcification (50%) and prosthetic endocarditis (27%). In a significantly smaller number of cases (15.1%), reoperations are performed due to the development of primary tissue failure [3].

In a number of studies, active participation of recipient cells in the formation of both calcium-associated damage to the implanted xenogenic material and degenerative changes in the structure of BHV caused by exposure to infectious agents was demonstrated [4, 5]. In addition, identification of various types of cells in the functionally preserved BHV [6, 7] suggests permanent remodeling of xenotissue after its implantation in the body, which implies a parallel course of its disintegration and repair processes [7]. On the one hand, this gives grounds to consider calcification as the final stage of the BHV destruction, which inevitably arises over time. On the other hand, it does not exclude the implementation of a fundamentally different scenario for the development of aseptic structural dysfunctions of implanted BHV depending on the type of remodeling.

The aim of this work was to study morphologically the surface and cellular composition of the leaflets of uncalcified BHV with varying degrees of xenotissue damage in order to identify possible mechanisms of development of primary tissue failure.

MATERIALS AND METHODS

Six xeno-aortic BHV models were studied: KemKor (n = 2) and PeriKor (n = 4), (Neokor CJSC, Kemerovo). They were preserved with ethylene glycol diglycidyl ether and removed from the mitral position during repeated surgical interventions. The removal took place due to the development of structural failure of BHV tissues without mineral inclusion deposition as computed tomography showed. Considering the differences in the implanted valve functioning caused by the influence of hemodynamic loads of different strengths [1, 8], only BHVs removed from the mitral position were included in the study. The group of reoperated patients consisted of 5 women and 1 man. The average age of patients at the time of repeated operations was 63.5 ± 4.8 years with an average BHV functioning duration of 7.3 ± 3.1 years.

For histological examination and immunohistochemistry assay (IHC), the whole BHV was placed in a 4% solution of paraformaldehyde for 48 hours. After fixation, fragments of valve leaflets were cut out for subsequent dehydration and embedding into Histomix paraffin mixture (BioVitrum, Russia). Sections (5 μm) were prepared from paraffin blocks on a semi-automatic rotary microtome (MZP 01 – Tekhnom, Russia). The sections were mounted on glass slides with a poly-L-lysine coating (Thermo Scientific, USA). The sections of BHV were stained with hematoxylin – eosin, and an IHG study was also performed. Verification of the absence of calcium in explanted BHV was carried out by staining with alizarin red.

IHC typing of cells was performed using the following markers: CD3 (T-lymphocytes), CD20 (B-lymphocytes), CD31, CD34 (hematopoietic progenitor cells), CD68 (monocytes / macrophages), vimentin (mesenchymal cells), and α- smooth muscle actin (smooth muscle cells). We used monoclonal mouse (CD3, CD20, CD34, CD68, vimentin) and rabbit (CD31, α-smooth muscle actin) antibodies produced by Novocastra Laboratories, Thermo Scientific and Spring Bioscience, which react with human antigens.

To identify the markers described above, high-temperature antigen unmasking was performed in a citrate buffer (0.01 M, pH 6.0) for α-smooth muscle actin, CD68, CD31, CD34, CD3; in a Tris-EDTA buffer (pH 9.0) for vimentin; CD20 – without unmasking. Endogenous peroxidase blocking, dilution of primary antibodies and their exposure time were determined according to the protocols of the primary antibody manufacturers. To detect the results of IHC reactions, the Novolink Polymer Detection System (Novocastra, UK) was used. The enzyme immunoassay was stopped by washing the sections in a phosphate buffer (pH 7.4), after which they were stained with Mayer hematoxylin and enclosed in a mounting medium. In parallel with the detection of antigens at each IHC staining, the positive and negative controls were set up. A negative control was carried out by applying 50 μl of antibody dilution solution to the sections (Ab Diluent, USA). A positive control of CD3, CD20, and CD68 markers was performed on sections of the human palatine tonsil, a positive control of CD31, CD34, vimentin and α-smooth muscle actin was carried out on sections of the human radial artery. The study of drugs and photography was performed using an AXIO Imager A1 microscope (Carl Zeiss, Germany) and a Canon G5 digital camera (Canon, Japan).

RESULTS

The degree of the BHV leaflet structure deterioration was significantly different, which allowed us to conditionally distinguish the following groups: samples with the intact endothelial layer on the surface of the BHV leaflet; samples with minimal or moderate disruption of the endothelial layer structure; and samples with severe endothelial cover destruction of the BHV leaflets, which extended into the leaflet and was accompanied by the destruction of its extracellular matrix.

In samples with the intact endothelial layer, a monolayer of cells morphologically corresponding to endotheliocytes was observed on the atrial and ventricular surfaces of the BHV leaflets. On the ventricular surface (excretory region), the monolayer was represented by flattened cells with elongated nuclei (Fig. 1*a*). On the atrial surface, the cells had rounded nuclei and a more pronounced cytoplasm (Fig. 1*b*). The layer of endotheliocytes on the ventricular surface appeared thinner than on the atrial one. An IHC assay of the BHV leaflets revealed CD31-positive staining of flat cells on both surfaces, confirming their endothelial phenotype (Fig. 1*b*). The absence of positive staining for CD34, in turn, indicated the maturity of endothelial cells (Fig. 1*g*).

BHV stroma was represented by compact, tightly packed bundles of collagen fibers that retained intact tortuosity and tinctorial properties (Fig. 1*a*, *b*). Moreover, a dense arrangement of fibers was observed in the surface layers, and loose arrangement – in deep layers. In some areas, small cavities with transparent contents were present (Fig. 1*a*, *b*).

Violation of the endothelial layer integrity with the presence of sections containing morphologically different cells, which form multilayer or single layer structures, was observed in samples with minimal or moderate damage to the BHV leaflets (Fig. 2). In some of the studied samples, cell infiltration of the underlying connective tissue structures of the BHV leaflet was also noted (Fig. 2*b*). In this case, the stroma of the BHV leaflets was characterized by moderate heterogeneity: in the surface layers – by loosening and thinning of bundles of collagen fibers with expansion of interfibrillar spaces and formation of mesh networks, in the deep layers – by relative intactness of the extracellular matrix (Fig. 2*a*, *b*).

In the zones of fibrous structure disorganization, the presence of CD68-positive cells belonging to the mononuclear phagocyte system was detected (Fig. 2*b*, *d*). Among them, in addition to typical macrophages,

individual multinuclear cells (Pirogov – Langhans cells) were identified. Vimentin-positive cells located singly or in groups were also found in the surface layers of the extracellular matrix, mainly in the areas of endothelial monolayer disturbance, which indicated that they belong to cells of the mechanocyte line.

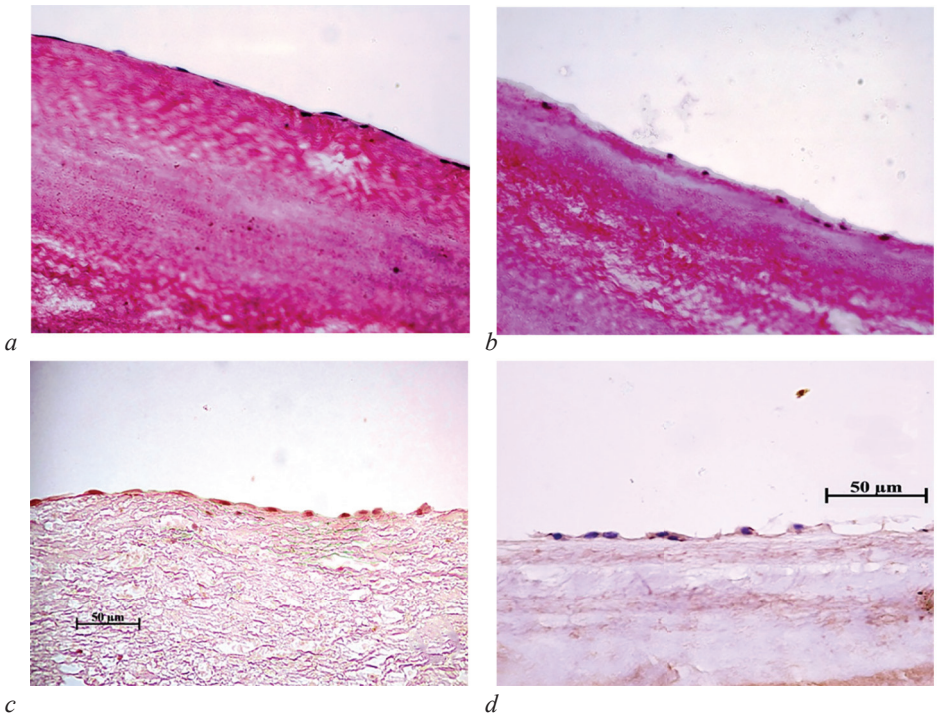


Fig. 1. The structure of the leaflets of bioprosthetic heart valves in areas with minimal damage to their structure, magnification 200. *a* – endothelium of the ventricular surface, *b* – endothelium of the atrial surface (stained with hematoxylin and eosin), *c* – IHC on CD31, *d* – IHC on CD34

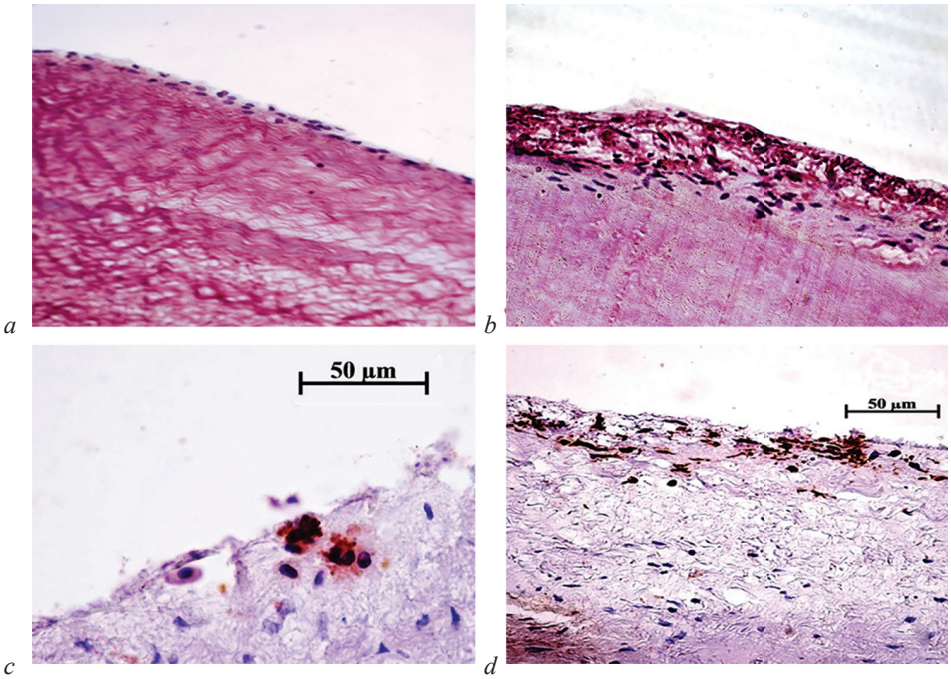
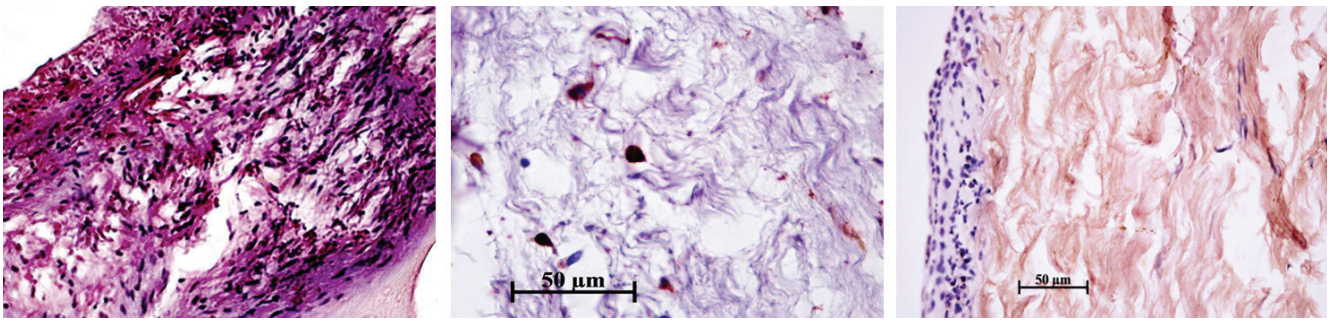


Fig. 2. The structure of the bioprosthetic heart valve leaflets with moderate damage. *a*, *b* – staining with hematoxylin and eosin, magnification 200, *c* – IHC on CD68, magnification 400, *d* – IHC in vimentin, magnification 200

In samples with pronounced destruction of the BHV leaflet surface, the absence of a monolayer of endotheliocytes was noted, which was associated with deep disorganization of their connective tissue base (Fig. 3). Stratification of collagen fiber bundles was combined with their fragmentation and formation of numerous cavities (Fig. 3*a*). Moreover, the entire thickness of the leaflet was infiltrated by cells. In the zones of the greatest destruction, both CD 68-positive and α-smooth muscle actin-positive cells were detected. CD68+ cells were mostly grouped around the cavities, adjacent to the remains of collagen fibers (Fig. 3B, *c*). Also, isolated smooth muscle cells were found in the thickness of the BHV leaflets, among the destroyed connective tissue structures (Fig. 3*c*).

It should be noted that in all three groups of samples, no positive IHC staining for T- and B-lymphocytes was observed.



a *b* *c*

Fig. 3. The structure of bioprosthetic heart valve leaflets with pronounced destruction of their surface and stroma. *a* – stained with hematoxylin and eosin, magnification 200, *b* – IHC on CD68, magnification 400, *c* – IHC on α-smooth muscle actin, magnification 200

DISCUSSION

The value of the presented morphological data for understanding the mechanisms of the degenerative changes in implanted BHVs in the recipient's organism primarily consists in the absence of infection and calcification. Thus, already at the stage of the study group formation, BHV dysfunctions were excluded, the occurrence of which is determined by the features of the immunological and metabolic status of patients leading both to a decrease in the microbial resistance of the biomaterial and its pronounced mineralization [1]. The results of the study suggest that various degrees of damage to the extracellular matrix can be considered as successive stages of destruction of BHV leaflets.

At the initial stage of tissue failure development, that is, in samples with intact structure, both surfaces were covered with a continuous layer of mature endothelium (CD31 +). Moreover, the morphological characteristics of endothelial cells had some differences. From the outflow side, endotheliocytes were characterized by a flattened form, a thin layer of cytoplasm and elongated nuclei with a heterochromatin predominance. On the inflow side, endothelial cells were higher than on the atrial surface, had round nuclei in which euchromatin predominated. Such differences in the endotheliocytes structure probably occur due to the influence of hemodynamic factors and may indicate different metabolic activities of these cells.

High degree of the extracellular matrix preservation suggested a possible protective function of the endothelium in relation to BHV damage by aggressive blood factors [5, 6]. At the same time, the presence of sites with collagen fiber delamination in these samples indicated the onset of destructive processes, presumably caused by prolonged cyclic deformations experienced by implantable BHV [9, 10].

Samples with minimal and moderate leaflet destruction were considered as the next stage in the development of primary tissue failure without mineralization of xenomaterial. It can be assumed that endothelial layer disintegration may trigger the development of hemodynamically significant damage to the structure of BHV. The causes of this process can be mechanical endotheliocyte destruction and exposure of extracellular matrix of valves and expression of endothelial cell adhesion molecules, which contribute to the attraction of monocytes with their subsequent migration deep into the BHV leaflets. The presented endothelial dysfunction can be triggered by various pathological processes [11], as well as by a low shear stress due to turbulent blood flow in the

absence of endothelium [12]. After differentiation of monocytes into macrophages, secretion of matrix metalloproteinases occurred, which led to the progression of destructive processes in the BHV leaflets and the formation of a pathophysiological "vicious circle" [12, 13]. Apparently, the process of cell differentiation was influenced by the microenvironment and the depth of its invasion in the BHV leaflets. For example, fibroblasts were localized mainly near the leaflet surface, and smooth muscle cells, as a rule, were present in the deeper layers of the leaflets.

It should be noted that at this stage of primary tissue failure development, a parallel course of extracellular matrix repair is not ruled out. Thus, the identification of mechanocyte cells, such as fibroblasts (vimentin-positive cells), indicates the possibility of synthesizing the main components of the leaflet extracellular matrix, which is aimed at replacing the degeneratively altered xenotissue [6]. However, progressive destructive processes indicate the predominance of fracture processes over reparation in the xenogenic material of the valves. The low regeneration rate can be caused not only by an insufficient number of fibroblasts, but also by their inability to fully function in atypical microenvironment conditions [14, 15]. In particular, under adverse conditions, a change in the functional properties of smooth muscle cells and fibroblasts and development of their destructive potential can occur [16–18]. This assumption is confirmed by the localization of these cells in the immediate vicinity of large cavities.

The absence of T- and B-lymphocytes in the studied samples, on the one hand, may indicate an insignificant role of inflammation in the development of the described variant of structural dysfunctions in primary tissue failure of BHV. On the other hand, it may suggest that calcification of chemically modified xenotissue in the recipient's body can only be realized under the conditions of immune inflammation activation.

CONCLUSION

The structure of the BHV explanted due to the primary tissue failure is characterized by impaired integrity or complete absence of an endothelial monolayer in the areas of extracellular matrix disintegration, as well as by the absence of lymphocytes. Thus, it can be assumed that it is the disintegration of the endotheliocyte layer that is the trigger for the primary tissue failure development. In other words, the optimal design of BHV should ensure adhesion and viability of endothelial cells on the leaflet surfaces in order to ensure the extracellular matrix integrity.

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Authors contribution

Mukhamadiyarov R.A. – conception and design of the study, analysis of the data, drafting of the manuscript. Rutkovskaya N.V. – analysis of the data, drafting of the manuscript. Kutikhin A.G. – drafting of the manuscript. Milto I.V. – carrying out of the immunohistochemistry assay, analysis of the data, drafting of the manuscript. Sidorova O.D. – analysis of the data. Barbarash L.S. – drafting of the manuscript.

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Morphological and functional characteristics of retrosternal adipose tissue and their relation to arterial stiffness parameters in patients after coronary artery bypass grafting

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ABSTRACT

Background. The attention of many researchers is focused on studying the role of adipokines secreted by subcutaneous, visceral, epicardial, and perivascular adipose tissues in the pathogenesis of diseases of the cardiovascular system. At the same time, adipose tissue of retrosternal localization remains out of research focus. This pool of fat cells is formed at the site of the thymic involution and has a significant volume. However, their functional activity and participation in the development of cardiovascular pathology remain unexplored.

Aim. To study the morphological characteristics of adipocytes of the retrosternal adipose tissue (RSAT) and their production of adipokines in comparison with epicardial (EAT) and subcutaneous adipose tissue (SCAT) and to investigate their relationships with arterial stiffness parameters in patients who underwent coronary artery bypass grafting.

Materials and methods. The study included 17 patients (12 men / 5 women aged 40 – 70 years) with the diagnosed coronary artery disease (CAD) who underwent coronary artery bypass grafting (CABG). Each patient underwent measurement of carotid-femoral pulse wave velocity (PWV) and aortic augmentation index (AIx) with the oscillometric device. Isolated adipocytes were obtained enzymatically from explants of SCAT, EAT and RSAT during coronary artery bypass grafting. The adipocytes were analyzed under the microscope at magnification 200. The release of adiponectin, leptin and insulin was studied in the adipocyte supernatant after 1 hour incubation using ELISA.

Results. It was found that adipocytes of the RSAT are smaller than adipocytes of SCAT: 83.96 ± 2.21 vs 98.62 ± 2.67 μm ($p = 0.00002$), respectively, and comparable in size to adipocytes of EAT: 86.65 ± 1.33 μm. The release of adiponectin by adipocytes of the RSAT turned out to be comparable to the production of this adipokine in SCAT and EAT, however, adipocytes of the RSAT produce less leptin than SCAT and EAT: 0.26 (0.19; 0.27) ng/l vs 0.37 (0.28; 0.55) ($p = 0.01$) and vs 0.32 (0.28; 0.44) ($p = 0.006$) ng/ml, respectively. Furthermore, RSAT produce less insulin than SCAT and EAT: 1.56 (1.03; 2.08) vs 1.70 (0.99; 2.18) ng/ml, ($p = 0.0022$) and 1.76 (1.16; 2.40) ng/ml ($p = 0.006$), respectively.

A positive correlation was found between the secretion of leptin by adipocytes of the RSAT and the AIx ($r_s = 0.52$, $p = 0.046$). An inverse relationship was found between insulin secretion by retrosternal adipocytes and PWV ($r_s = –0.55$, $p = 0.035$). There was no relationship between the size of the retrosternal adipocyte or hypertrophy of the thymic adipocytes (more than 100 μm) and the production of leptin and insulin and arterial stiffness parameters.

Conclusions. The data of our pilot study show that adipocyte hypertrophy of the retrosternal AT is not a significant marker of adipokine production disturbance. The observed relationships suggest that an increase in leptin production and reduced insulin secretion by retrosternal AT may contribute to the formation of adipokine-related

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arterial stiffness. Based on the data obtained, it can be assumed that adipokines produced by the retrosternal AT can participate in the formation of arterial stiffness in patients with coronary artery disease.

Key words: epicardial, retrosternal and subcutaneous adipose tissue, arterial stiffness, adipocyte, adipokines, coronary artery disease.

Conflict of interest. The authors declare no 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 performed in accordance with the Helsinki Declaration of the World Medical Association "Ethical Principles for Conducting Scientific Medical Research with Human Participation" as amended in 2000 and the "Rules of Clinical Practice in the Russian Federation" approved by the Order No. 266 of the Ministry of Health of the Russian Federation of June 19, 2003. The research was approved by the local Ethics Committee of the Cardiology Research Institute of Tomsk National Research Center (Protocol No.146 of 16.06.2016). All individuals included in the study signed an informed consent.

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Морфофункциональные характеристики загрудинной жировой ткани и их связь с артериальной жесткостью у пациентов с коронарным атеросклерозом, подвергшихся операции аортокоронарного шунтирования

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РЕЗЮМЕ

Цель – исследование морфофункциональных характеристик адипоцитов загрудинной жировой ткани (ЗЖТ) в сравнении с другими типами жировой ткани (ЖТ), а также изучение их связи с показателями артериальной жесткости у пациентов с коронарным атеросклерозом, подвергшихся операции аортокоронарного шунтирования (АКШ).

Материалы и методы. В настоящее пилотное исследование включены 17 пациентов (12 мужчин и 5 женщин) в возрасте 40–70 лет со стабильной ишемической болезнью сердца и документированным коронарным атеросклерозом, которым была проведена операция АКШ и которые подписали информированное согласие на участие в исследовании. Материалом для исследования явились эксплантаты эпикардиальной, подкожной и загрудинной жировой ткани (ЗЖТ), их забор осуществлялся в ходе операции. Для изучения состояния регионарной артериальной жесткости использовали осциллометрическую артериографию (TensioMed, Венгрия). Определяли уровень адипонектина, лептина, инсулина в супернатантах адипоцитов.

Результаты. Обнаружено, что адипоциты ЗЖТ имели меньшие размеры, чем адипоциты подкожной ЖТ, и были сопоставимы по размеру с эпикардиальными адипоцитами. Выброс адипонектина адипоцитами ЗЖТ не имел различий с таковым в подкожной и эпикардиальной ЖТ, однако адипоциты ЗЖТ вырабатывали существенно меньше лептина и инсулина. Впервые продемонстрирована взаимосвязь выработки адипоци-

тами ЗЖТ лептина и инсулина с показателями регионарной артериальной жесткости: прямая корреляционная связь – между секрецией лептина адипоцитами ЗЖТ и аортальным индексом аугментации и обратная – между секрецией инсулина адипоцитами ЗЖТ и скоростью пульсовой волны. Линейных корреляций между размерами адипоцитов ЗЖТ, наличием адипоцитов >100 мкм ЗЖТ и выработкой адипоцитами ЗЖТ лептина, инсулина, а также параметрами регионарной артериальной жесткости выявлено не было.

Заключение. Продемонстрировано наличие у ЗЖТ секреторной активности, интенсивность которой не имеет линейных ассоциаций с размером адипоцитов и их гипертрофией. Полученные нами данные впервые указывают на взаимосвязь адипокинов, вырабатываемых ЗЖТ, с процессами нарушения эластических свойств магистральных артерий у пациентов с коронарным атеросклерозом.

Ключевые слова: эпикардиальная, загрудинная и подкожная жировая ткань, артериальная жесткость, адипоциты, адипокины, коронарный атеросклероз.

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

Источник финансирования. Статья подготовлена в рамках темы фундаментальных исследований № АААА-А15-115123110026-3.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование было одобрено локальным этическим комитетом НИИ кардиологии Томского НИМЦ (протокол № 146 от 16.06.2016).

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INTRODUCTION

Currently, the attention of a large number of researchers is focused on studying the role of adipokines secreted by visceral adipose tissue and its ectopic depots in the pathogenesis of cardiovascular diseases [1–3]. Numerous studies have shown the pathological role of epicardial obesity, while adipose tissue of the retrosternal localization remains out of research focus. This pool of adipose cells has a significant volume and is formed mainly due to age-related involution of the key organ of the immune system – the thymus, when it is almost completely replaced by adipose tissue [4]. The morphological characteristics of adipocytes of the retrosternal adipose tissue (RSAT), their functional activity and possible participation in the development of cardiovascular pathology have not been studied yet. However, it is the structural and functional features of fat depots that are the most important pathological factor in development of a high cardiometabolic risk.

It is known that both obesity and arterial stiffness are independent predictors of cardiovascular morbidity and mortality [5]. Recent publications have reported a close relationship between the epicardial fat depots and an increase in arterial stiffness, which is presumably associated with adipocyte dysfunction and impaired adipokine production [6], while there is no information on this with respect to RSAT.

The aim of this work was to study the morphological and functional characteristics of RSAT adipocytes in comparison with other types of adipose tissue (epicardial and subcutaneous) as well as to investigate their potential relationship with arterial stiffness in patients with coronary atherosclerosis after coronary artery bypass grafting (CABG).

MATERIALS AND METHODS

The present pilot study included 17 patients (12 men and 5 women) aged 40-70 years with stable coronary artery disease (CAD) and documented coronary atherosclerosis, who had indications for CABG.

The study was performed in accordance with the Helsinki Declaration of the World Medical Association “Ethical Principles for Conducting Scientific Medical Research with Human Participation” as amended in 2000 and the “Rules of Clinical Practice in the Russian Federation” approved by the Order No. 266 of the Ministry of Health of the Russian Federation of June 19, 2003. The research was approved by the local Ethics Committee of the Cardiology Research Institute of Tomsk National Research Center (Protocol No. 146 of 16.06.2016). All individuals included in the study signed an informed consent.

All patients received regular drug therapy. The proportion of smokers and patients with metabolic disorders that met the criteria for the metabolic syndrome [7] was high. The clinical characteristics of patients are presented in Table 1.

Exclusion criteria were acute atherosclerotic complications over the past 6 months; any inflammatory disease; chronic kidney disease above C3b; and oncological, hematological and immune diseases.

Table 1

Clinical and demographic characteristics of the studied patients (n = 17)

Parameters	
Gender	12/5
Age, years old	63 (59;66)
History of myocardial infarction, *n* (%)	6 (35.3)
Arterial hypertension, *n* (%)	15 (88.2)
Diabetes mellitus, *n* (%)	4 (23.5)
Duration of arterial hypertension, years, *Me* (Q_{25}; Q_{75})	15 (10; 20)
Duration of coronary artery disease, years, *Me* (Q_{25}; Q_{75})	2 (1.75; 5.5)
Systolic blood pressure, mm Hg, *Me* (Q_{25}; Q_{75})	135 (127; 142)
Diastolic blood pressure, mm Hg, *Me* (Q_{25}; Q_{75})	77.5 (69.5; 84.5)
Smoking, *n* (%)	11 (64.7)
Body mass index, kg/m^2, *Me* (Q_{25}; Q_{75})	29.4 (28.1; 31.2)
Obesity, *n* (%)	8 (47)
Waist circumference, cm	103 (92; 110)
Note: *Me* [Q_1;Q_3]	

All patients underwent selective coronary angiography on the Cardioscop-V angiographic complex and Digitron-3NAC computer system, Siemens (Germany), at the Department of X-ray Diagnosis and Treatment (supervisor – Bayev A.E., Cand. Sci. (Med.)). Anthropometric measurements were performed to assess total obesity according to body mass index (BMI) and abdominal obesity according to the waist circumference. Oscillometric arteriography (TensioMed, Hungary) was used to study the state of regional arterial stiffness. The pulse wave propagation velocity (PWV) and aortic augmentation index were evaluated.

The material for the study was explants of retrosternal (RSAT), subcutaneous (SCAT) and epicardial (EAT) tissues weighing 0.5–1 g. The explants were collected during CABG. The samples were placed in M199 medium and delivered to the laboratory within 15 minutes. The adipose tissue cells were isolated enzymatically, in sterile conditions of the laminar cabinet of the II protection class (BAVp-01 – Laminar-s – 1.5, Laminar systems, Miass, Russia) [8]. The tissue was minced, incubated for 35–40 min at a temperature of 37 ° C and constant gentle stirring (10 rpm) in 5 ml of a sterile solution of type I collagenase (PanEco, Russia) 1 mg/ml in the Krebs – Ringer buffer (2 mM D-glucose, 135 mM NaCl, 2.2 mM CaCl2 · 2H2O, 1.25 mM MgSO4 · 7H2O, 0.45 mM KH2PO4, 2.17 mM Na2HPO4, 25 mM HEPES, 3.5% BSA, 0.2 mM adenosine). To neutralize collagenase, the Krebs – Ringer buffer was added in a 1:1 ratio. The cell suspension was filtered through a nylon filter (Falcon ™ Cell strainer, pore diameter 100 μm), and washed three times with warm Krebs – Ringer buffer. In each sample, 200–600 cells in total were analyzed in non-overlapping visual fields using light microscopy at magnification 200 (Axio Observer Z1 microscope, Carl Zeiss Surgical GmbH, Germany) (Fig. 1). The number and size of the adipocytes were calculated. Adipocytes > 100 μm were classified as hypertrophic. Adipocytes in the amount of 20 × 10^5 were added to a well of a sterile 24-well plate (Greiner, Germany); the volume of the well was adjusted to 1 ml and incubated for 1 hour at 37 °C with constant stirring at 10 rpm.

a *b* *c*

Fig. 1. Light microscopy of isolated adipocytes of subcutaneous (*a*), epicardial (*b*) and retrosternal (*c*) adipose tissue. Magnification 200

Adipocyte supernatants were collected from the bottom of the wells, frozen and stored at –70°C.

The adiponectin ELISA kit (Mediagnost, Germany) was used to determine adiponectin in adipocyte supernatants, the Leptin Sensitive ELISA kit (Mediagnost, Germany) was used to determine leptin; insulin was determined using the Insulin Test System kit (Monobind Inc.; USA)

Statistical analysis was performed using the Statistica 10.0 software (StatSoft Inc., USA). The data distribution was tested the by Shapiro – Wilk test. The median and interquartile range of the 25th and 75th percentiles were used to describe data with non-standard distribution. A mean and a standard error of the mean (*M* ± *SEM*) were used for description of data with standard distribution. The differences between groups with non-standard distribution were determined according to the Wilcoxon signed rank test. In cases with standard data distribution, the paired sample t-test was used. To evaluate the relationship between parameters, the Spearman's rank correlation coefficient (*Rs*) was used. All statistical hypotheses were accepted at $p < 0.05$.

RESULTS

A morphometric study showed no difference in the size of adipocytes of RSAT and EAT, while the size of adipocytes of RSAT was significantly smaller than that of SCAT (Table 2). The part of hypertrophied adipocytes, defined as the percentage of cells >100 μm, was more than two times less in the RSAT than in the SCAT. The part of hypertrophied adipocytes of EAT did not differ from the same indicator for RSAT, and the part of small adipocytes (< 50 microns) in all three tissues was comparable. For RSAT, this indicator amounted to 2.23 (1.08; 4.40) %, for EAT – 2.07 (0.83; 3.87)%, and for SCAT – 1.92 (0.23; 4.68)%. Therefore, RSAT adipocytes are smaller and less hypertrophied than SCAT adipocytes. At the same time, adipocytes of RSAT are comparable in size to epicardial adipocytes. The size of RSAT adipocytes did not correlate with the body mass index.

Table 2

The size of adipocytes in subcutaneous, epicardial and retrosternal adipose tissue

Parameters	Subcutaneous adipose tissue	Epicardial adipose tissue	Retrosternal adipose tissue
Adipocyte size, μm, *M* ± *SEM*	98.62 ± 2.67	86.65 ± 1.33	83.96 ± 2.21 $p_1 = 0.00002$ $p_2 = 0.23$
Percentage of hypertrophied adipocytes, %, *Me* (Q_{25}; Q_{75})	47.66 (31.78; 55.50)	17.59 (9.84; 25.53)	16.11 (11.73; 30.20) $p_1 = 0.000062$ $p_2 = 0.81$

Note. Adipocytes >100 μm were considered hypertrophied; p_1 – comparison of retrosternal adipose tissue with subcutaneus adipose tissue, p_2 – comparison of retrosternal adipose tissue with epicardial adipose tissue (paired sample t-test); p_1 – comparison of retrosternal adipose tissue with subcutaneous adipose tissue, p_2 – comparison of retrosternal adipose tissue with epicardial adipose tissue (using Wilcoxon signed-rank test).

Adiponectin release by adipocytes of RSAT was found to be comparable with its production by adipocytes of EAT and SCAT (Table 3). It was found that the level of leptin in the incubation medium of RSAT adipocytes was 30% and 20% lower than this indicator for SCAT and EAT, respectively.

Table 3

Production of adipokines by adipocytes of subcutaneous, epicardial and retrosternal adipose tissue, *Me* (Q_{25}; Q_{75})

Parameters	Subcutaneous adipose tissue	Epicardial adipose tissue	Retrosternal adipose tissue
Adiponectin, ng/ml	10.59 (8.31; 12.25)	7.93 (6.77; 10.11)	10.21 (8.77; 12.78)
Leptin, ng/ml	0.37 (0.28; 0.55)	0.32 (0.28; 0.44)	0.26 (0.19; 0.27) $p_1 = 0.009$ $p_2 = 0.006$
Leptin/adiponectin	0.038 (0.028; 0.069)	0.033 (0.019; 0.044)	0.022 (0.019; 0.028) $p_1 = 0.001$ $p_2 = 0.004$
Insulin, ng/ml	1.70 (0.99; 2.18)	1.76 (1.16; 2.40)	1.56 (1,03; 2.08) $p_1 = 0.002$ $p_2 = 0.006$

Note. p_1 – comparison of retrosternal adipose tissue with subcutaneus adipose tissue, p_2 – comparison of retrosternal adipose tissue with epicardial adipose tissue (using Wilcoxon signed rank test).

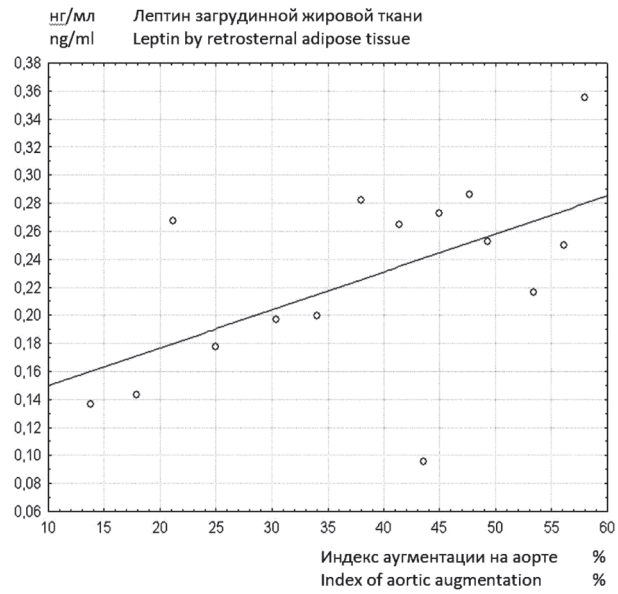

Fig. 2. Correlation between the production of leptin by retrosternal adipose tissue adipocytes and index of aortic augmentation, $r_s = 0.52$, $p = 0.047$

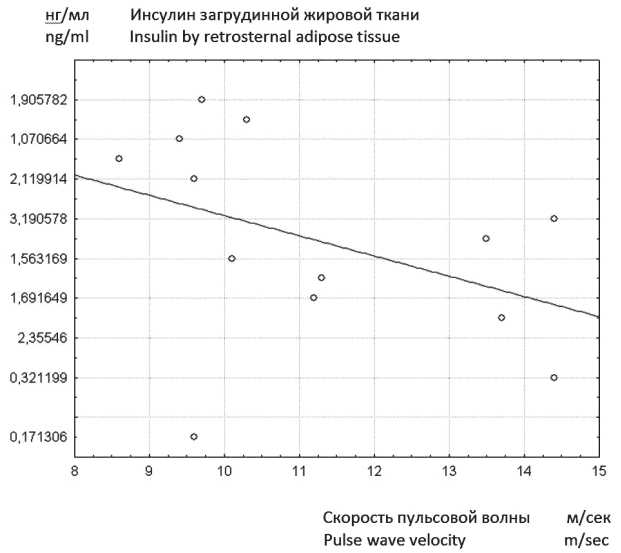

Fig. 3. Correlation between the production of insulin by retrosternal adipose tissue adipocytes and pulse wave velocity, $r_s = –0.55$, $p = 0.035$

Since there were no significant differences in the production of adiponectin by the studied adipose tissue, the RSAT leptin/adiponectin index was one and a half times lower than for SCAT and EAT. The production of insulin by adipocytes of RSAT exceeded that by 8% ($p = 0.022$) for SCAT and by 11% ($p = 0.006$) for EAT.

During correlation analysis, we first discovered the relationship between the secretion of leptin and insulin by RSAT adipocytes and the parameters of regional arterial stiffness. A direct correlation between the secretion of leptin by RSAT adipocytes and the aortic augmentation index was observed (Fig. 2). An indirect relationship was observed between the secretion of insulin by RSAT adipocytes and the pulse wave propagation velocity, PWV (Fig. 3). At the same time, we did not reveal a linear relationship between the sizes or hypertrophy of RSAT adipocytes and the production of leptin, insulin and the leptin / adiponectin ratio.

DISCUSSION

The relationship between the accumulation and dysfunction of visceral adipose tissue and the risk of developing cardiovascular disease was confirmed in numerous studies [9–11]. The key factors of this pathological chain are the excess production of adipokines and reactive oxygen species by adipose tissue [2, 3, 12, 13]. A relationship between dysfunction of adipocytes of small fat depots – perivascular and epicardial localization – and the development of atherosclerosis [14, 15, 16], impaired arterial elasticity [6], arterial hypertension [17], heart failure, and arrhythmia was found [18]. An association of visceral obesity and increased leptin production in adipose tissue with the systemic hypertension and increased arterial stiffness was reported [19–21], while the retrosternal fat depots formed as a result of age-related thymic involution remain out of focus of investigators.

In our work, the morphological characteristics of adipocytes of retrosternal adipose tissue and their production of insulin and adipokines in patients with coronary atherosclerosis who underwent CABG were studied for the first time. According to the results of our study, the size of adipocytes of RSAT corresponds to that in EAT. Our data do not confirm the association of RSAT adipocyte size with body mass index or the intensity of adipokine production by them. That does not allow us to consider RSAT adipocyte hypertrophy as a significant marker of impaired adipokine production. The lack of correlation between the size of adipocytes and BMI has been previously shown for epicardial adipocytes and, possibly, is typical for small fat depots [22]. At the same time, in the literature, there are few data on the correlation between the

size of EAT adipocytes and the leptin and adiponectin level in blood serum [23]. There is no information on the ratio of the size/production of adipokines by EAT adipocytes. Thus, the current opinion about the dependence of the adipocyte secretory activity on its hypertrophy for small fat depots requires additional factual evidence.

Our results showed that the intensity of adiponectin secretion by the RSAT cells does not differ from that in EAT and SCAT, which makes it possible to consider RSAT as a secretory organ. Nevertheless, the production of leptin and insulin by RSAT adipocytes, according to our data, turned out to be significantly lower than in EAT and SCAT. This fact may be associated with the formation of RSAT on the site of a previously functionally active immune regulatory organ – the thymus, which underwent age-related involution.

We found a direct correlation between the production of leptin by RSAT and the aortic augmentation index. An indirect relationship between the production of insulin in RSAT and the pulse wave velocity was observed. Considering the mechanism of the leptin influence on the state of arterial stiffness, it can be assumed that it can be realized through the profibrotic effect of leptin. In an experimental study on a model of isolated smooth muscle cells derived from the aorta of obese rats, it was found that the addition of leptin to the incubation medium led to an increase in the collagen II gene expression, a rise in the cellular content of collagen, fibronectin, a transforming growth factor (TGFβ) and connective tissue growth factor (CTGF) [24]. In addition, the ability of leptin to induce hypertrophy of vascular smooth muscle cells, their osteogenic differentiation and expression of metalloproteinases was reported [25].

Our data on the indirect relationship of insulin produced by RSAT adipocytes with arterial stiffness are consistent with the published data. Thus, experimental studies have shown a decrease in the elastic properties of the aorta in insulin-resistant rats [26] and in rats with diabetes [27]. This fact is confirmed by the results of clinical studies on the inverse dependence of arterial stiffness parameters (pulse wave velocity) on the dosage of insulin administered to patients with metabolic syndrome and type 2 diabetes mellitus [28]. Among the mechanisms mediating the effect of insulin on the elastic properties of the arterial wall, one can consider its effects on the intracellular protective mechanism, including the phosphorylation of Akt, ERK-1/2 and JNK-1/2 kinases followed by activation of the hypoxia-induced factor 1α (HIF-1α) [29], as well as the suppressive effect of insulin on nitric oxide synthase type II (eNOS) [30].

CONCLUSION

Thus, the results of this pilot study demonstrate the presence of secretory activity in the retrosternal adipose tissue, the intensity of which does not have a linear relationship with the size of adipocytes and their hypertrophy. Our data revealed an association between the production of adipokine in the retrosternal adipose tissue and deterioration of the elastic properties of arteries in patients with coronary atherosclerosis.

The limitations of the study are its small volume and a lack of separate studies on the morphological and functional features of RSAT in men, women and patients with diabetes mellitus and obesity.

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Naryzhnaya N.V. – conception and design, collection of experimental material, analysis and interpretation of data, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Koshelskaya O.A. – conception and design, analysis and interpretation of data, substantiation of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Kharitonova O.A. – collection of clinical material, analysis and interpretation of data. Zhigaleva N.I. – collection of experimental material, analysis and interpretation of data. Zhuravleva O.A. – analysis and interpretation of data, critical revision of the manuscript for important intellectual content. Evtushenko V.V. – collection of clinical material, substantiation of the manuscript. Andreev S.L. – collection of clinical material, substantiation of the manuscript. Evtushenko A.V. – conception and design, collection of clinical material. Boshchenko A.A. – conception and design, final approval of the manuscript for publication.

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Molecular mechanisms of the effects of N-ethylmaleimide and 1,4-dithioerythritol on regulation of apoptosis in P19 cells under hypoxia

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ABSTRACT

Impairment of apoptosis regulation in P19 cells is correlated with generation of oxidative stress. Under hypoxia, changes in mitochondrial functions occur, which may exacerbate oxidative stress in the tumor cell.

The aim of the study was to evaluate the effects of N-ethylmaleimide and 1,4-dithioerythritol on implementation and regulation of apoptosis in P19 cells under hypoxia *in vitro*.

Materials and methods. P19 cells (mouse teratocarcinoma) cultured under hypoxia served as the material for the study. For redox status modulation, 5mM N-ethylmaleimide and 1,4-dithioerythritol in the final concentrations of 5 mM were used. The intracellular concentration of calcium ions, the transmembrane potential and the number of Annexin V, CD95 and CD120 positive cells were determined by flow cytometry. The levels of reduced, oxidized and protein-bound glutathione, protein SH groups, hydroxyl radical and protein carbonyl derivatives were measured by spectrophotometry.

Results. The alteration in the redox status of the glutathione system under hypoxia, accompanied by oxidative modification of proteins (glutathionylation and carbonylation), influences the metabolism in the tumor cell on the whole. Under the effects of 1,4-dithioerythritol, an SH group protector, this alteration promotes formation of additional mechanisms to escape apoptosis, whereas under the effects of N-ethylmaleimide, an SH group blocker, it, on the contrary, promotes apoptosis activation.

Conclusions. The changes in the redox homeostasis of the tumor cell and modulation of oxidative modification of proteins (glutathionylation and carbonylation) under hypoxia are one of the promising approaches to targeted regulation of cell death.

Key words: redox status, tumor growth, oxidative stress, glutathione system, apoptosis, hypoxia.

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Молекулярные механизмы влияния N-этилмалеимида и 1,4-дитиоэритритола на регуляцию апоптоза опухолевых клеток линии P19 при гипоксии

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РЕЗЮМЕ

Актуальность. Нарушение регуляции апоптоза в эпителиальных опухолевых клетках линии P19 сопряжено с формированием окислительного стресса. В условиях гипоксии происходит изменение функционирования митохондрий, что может выступать дополнительным фактором, усугубляющим окислительный стресс в опухолевой клетке.

Цель – оценить влияние N-этилмалеимида и 1,4-дитиоэритритола на реализацию и регуляцию апоптоза опухолевых клеток линии P19 при гипоксии *in vitro*.

Материалы и методы. Материалом для исследования служили культивированные в условиях гипоксии опухолевые клетки линии P19 (тератокарцинома мыши). Для модуляции редокс-статуса использовали N-этилмалеимид в концентрации 5 мМ и протектор SH-групп – 1,4-дитиоэритритол в конечной концентрации 5 мМ. Методом проточной цитофлуориметрии определяли внутриклеточное содержание ионов кальция, трансмембранный потенциал митохондрий, количество аннексин V-, CD95- и CD120-положительных клеток. Концентрацию восстановленного, окисленного и белково-связанного глутатиона, SH-групп протеинов, гидроксильного радикала и карбонильных производных белков измеряли методом спектрофотомерии.

Результаты. В условиях гипоксии изменение редокс-статуса системы глутатиона, сопровождающееся окислительной модификацией белков (глутатионилирование и карбонилирование), оказывает влияние на метаболизм опухолевой клетки в целом и, при применении протектора SH-групп белков – 1,4-дитиоэритритола, способствует формированию дополнительных механизмов ускользания от клеточной гибели, а в случае применения блокатора SH-групп протеинов – N-этилмалеимида – активации апоптоза.

Заключение. В условиях гипоксии изменение редокс-гомеостаза опухолевой клетки и модуляция окислительной модификации белков (глутатионилирование и карбонилирование) являются одним из перспективных подходов таргетной регуляции клеточной гибели.

Ключевые слова: редокс-статус, опухолевый рост, окислительный стресс, система глутатиона, апоптоз, гипоксия.

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INTRODUCTION

Steady growth of incidence and high mortality from cancer pose a challenge of finding molecular targets for the activation of tumor cell death to theoretical and practical medicine. A significant contribution to the change in the redox status of the cell is made by the functioning of mitochondria as the main source of reactive oxygen intermediate generation [1, 2]. It is known that the level of production of reactive oxygen intermediates directly depends on oxygen tension inside the cell and the activity of enzymes of the electron transport chain. Currently, reactive oxygen intermediates are considered as important signaling molecules that can cause oxidation of nucleic acids, lipids, and functional domains of proteins [3]. Oxidative modification of these macromolecules contributes to genome instability, uncontrolled proliferation, impairment of apoptosis regulation, activation of angiogenesis, and a change

in the direction of intracellular metabolic pathways of tumor cells [4–7]. Accumulation of oxidatively modified proteins in tumor cells is one of the aspects of proteasome activation and production of heat-shock proteins that are involved in the regulation of cell death [8–10]. The study of mechanisms that trigger tumor cell apoptosis under hypoxia is of particular interest, since the tumor cell acquires additional resistance not only to apoptotic death, but also to chemotherapy [11, 12].

From our point of view, glutathionylation and carbonylation of proteins are among promising molecular mechanisms for redox regulation of intracellular signaling and tumor cell death under hypoxia. Potential intracellular targets of reversible and irreversible modification of proteins may be key proteins that regulate the cell cycle, ion-transporting systems, and transcription factors [3, 13].

The aim of the study was to evaluate the effect of N-ethylmaleimide and 1,4-dithioerythritol on the implementation and regulation of apoptosis of P19 tumor cells under hypoxia *in vitro*.

MATERIALS AND METHODS

P19 tumor cells (C3H/He mouse teratocarcinoma) from the cell culture bank of the Institute of Cytology of the Russian Academy of Sciences (St.-Petersburg, Russia) served as the material for the study. The cells were cultured using the monolayer method in a CO_2 incubator (Sanyo, Japan) at a temperature of 37 °C in the 5% CO_2 atmosphere. Culture conditions were the following: αMEM culture medium (BioloT, Russia), 10% fetal bovine serum (BioloT, Russia), L-glutamine 0.3 mg/ml (BioloT, Russia) and gentamicin 100 μg/ml (Microgen, Russia). Cell viability was assessed using a 0.5% trypan blue solution (Serva, USA). A cell culture having no more than 5% of dead cells was used for the experiment.

For the purpose of additional production of reactive oxygen intermediates by tumor cells, hypoxia was simulated in a culture of P19 tumor cells in the Hypoxia Incubator Chamber (STEMCELL, Canada) using the following gas mixture: 5% O_2, 5% CO_2, 90% N_2. The creation of hypoxia conditions was monitored by measuring the concentration of dissolved oxygen using the Dissolved Oxygen Meter (HANNA HI 9146, Italy).

An SH group blocker – N-ethylmaleimide (NEM) (Sigma-Aldrich, USA) – at a concentration of 5 mmol [14] and an SH group protector – 1,4-dithioerythritol (DTE) (Sigma-Aldrich, USA) – at a concentration of 5 mmol [15] were used as redox status modulators.

After incubation, the tumor cells were washed from the culture medium and lysed by resuspension in phosphate-buffered saline (pH = 7.4) with the addition of 1% X-100 Triton (Sigma-Aldrich, USA). After that they were cooled down using ice, maintaining the standard cell concentration to determine the concentration of the hydroxyl radical and carbonyl derivatives of proteins. The content of the hydroxyl radical was determined through its ability [after preliminary opsonization of cells with zymosan (Sigma-Aldrich, USA)] to destroy the model substrate – 2-deoxy-D-ribose (Sigma-Aldrich, USA) – and form a reaction product with the maximum absorption at 532 nm [16]. The concentration of carbonyl derivatives of proteins was determined through their reaction with 2,4-dinitrophenylhydrazine, which product has the maximum absorption at a wavelength of 363 nm [17]. The results on the content of the hydroxyl radical and carbonyl derivatives of proteins were expressed in nmol/mg of protein.

To determine the content of reduced, oxidized, protein-bound glutathione and protein SH groups, the cell lysate was deproteinized using a 5% sulfosalicylic acid solution. The concentration of total, oxidized (GSSG) and reduced (GSH) glutathione was determined using the method proposed by M.E. Anderson modified by I. Rahman et al. [18]. The GSH/GSSG ratio was calculated as an indicator of the change in the cell redox status. The content of protein SH groups and protein-bound glutathione, after its preliminary release from binding with proteins using a 1% sodium borohydride solution, was determined based on the reaction with 5.5-dithiobis-(2-nitrobenzoic acid) [19]. The results on the content of fractions of glutathione, protein SH groups and protein-bound glutathione were expressed in nmol/mg of protein.

Protein content in the cells was determined using the Bradford method based on the interaction of amino acid residues of lysine and arginine with the Coomassie blue dye G-250 [20].

Extinction of results was determined using the SF-2000 spectrophotometer (OKB-Spektr, Russia).

Apoptotically modified cells were evaluated by flow cytometry using annexin-V-FITC and propidium iodide (PI) according to the manufacturer's instructions (eBioscience, USA). The number of Annexin V positive cells was counted with respect to the total number of studied cells and expressed in %.

The number of CD95 and CD120 positive cells was determined using a set of monoclonal antibodies to the corresponding antigens according to the manufacturer's protocol (R&D Systems, USA). The result was expressed in conditional units (cu).

The mitochondrial membrane potential ($\Delta\Psi m$) of the cells was evaluated with the help of the Flow Cytometry Mitochondrial Membrane Potential Detection Kit (BD, USA) for reduction of spectral luminescence using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolecarbocyanine iodide, which, upon depolarization

of the mitochondrial membrane, is unable to penetrate inside the organelles and form fluorescent aggregates. The number of cells with reduced fluorescence was expressed in %.

The concentration of calcium ions in cell cytoplasm was determined using the method based on their binding to the maximum fluorescence at 526 nm by the Fluo 3 AM lipophilic probe (Sigma-Aldrich, USA) [21]. The results were expressed in conditional units (cu) reflecting the level of probe luminescence per cell.

Flow cytometry results were detected using the FACSCanto II instrument (BD, USA) and FACSDiva Version 6.1.3 software.

Statistical processing of the obtained data was carried out using SPSS 17.0 software. The normality of distribution of quantitative indicators was checked using the Shapiro – Wilk criterion. The significance of differences was evaluated using the non-parametric Kruskal – Wallis and Mann – Whitney tests. The data were presented as a median (*Me*), upper and lower quartiles (Q_1–Q_3). The differences were considered statistically significant at $p < 0.05$.

RESULTS

The impairment of the regulation of apoptotic death of tumor cells against the background of a decrease in intracellular oxygen tension is associated with a change in their redox status. Excessive generation of reactive oxygen intermediates under hypoxia is associated with the loss of electrons from the mitochondrial respiratory chain due to the absence of the finite acceptor of electrons and a decrease in the activity of complex IV – cytochrome c oxidase. In this regard the main reason for the formation of oxidative stress inside the cell is the impairment of these organelles functioning [22]. The most toxic reactive oxygen intermediate for cell macromolecules is the hydroxyl radical. According to some authors, it is the $OH^{\bullet}$-radical that acts as the most powerful agent contributing to the oxidative modification of proteins [23, 24]. In the previous study on the effect of hypoxia on the metabolism of P19 tumor cells, we showed the formation of oxidative stress associated with a change in the state of the glutathione system and apoptosis activation, compared to tumor cells cultured at a normal oxygen concentration [25].

Under hypoxia, with the addition of the protein SH group protector (DTE) to the culture medium of P19 tumor cells, we obtained a decrease in the concentration of $OH^{\bullet}$-radical by 2.5 times ($p < 0.05$) as well as in the number of cells with reduced mitochondrial potential by 5.2 times ($p < 0.05$), compared with the results recorded in the cells under hypoxia (see the table). In addition, under hypoxia, the action of DTE was accompanied by a significant increase in the GSH content by 1.3 times ($p < 0.05$), in the concentration of free protein SH groups by 1.5 times ($p < 0.05$), and by a decrease in the content of protein-bound glutathione by 1.4 times ($p < 0.05$) against the background of a comparable concentration of carbonyl derivatives of proteins, GSSG and GSH/GSSG ratio, as opposed to cells cultured under hypoxia (see the table). When studying the implementation and regulation of apoptosis under conditions of hypoxia and addition of the protector of protein SH groups to the culture medium of P19 tumor cells, we obtained a significant decrease in the number of Annexin V positive cells by 2.0 times ($p < 0.05$) and in the intracellular content of Ca^{2+} ions by 1.1 times ($p < 0.05$) against the background of comparable values for CD95 and CD120 positive cells, as opposed to cells under hypoxia (see the table). So, 1,4-dithioerythritol contributed to the formation of additional resistance of P19 tumor cells to the mechanisms triggering apoptosis under conditions of hypoxia.

When modulating the redox status of P19 tumor cells using a protein SH group blocker (NEM) under hypoxia, we obtained a significant increase in the number of Annexin V positive cells by 9.1 times ($p < 0.05$), CD95 positive cells by 15.5 times ($p < 0.05$), CD120 positive cells by 2.9 times ($p < 0.05$), cells with a reduced mitochondrial potential by 8.8 times ($p < 0.05$) and the intracellular concentration of Ca^{2+} ions by 3.0 times ($p < 0.05$) against the background of a comparable value for the $OH^{\bullet}$-radical in comparison with the results recorded for the cells under hypoxia (see the table). The effect of NEM in P19 tumor cells under hypoxia was accompanied by a significant decrease in the concentration of GSH by 3.4 times ($p < 0.05$) and an increase in the concentration of GSSG by 1.6 times ($p < 0.05$), which led to a significant decrease in the GSH/GSSG ratio by 5.4 times ($p < 0.05$), compared with cells under hypoxia (see the table). In this case the effect of the blocker of protein SH groups in P19 tumor cells under hypoxia was accompanied by a significant increase in the concentration of protein carbonyl derivatives by 1.4 times ($p < 0.05$) against the background of comparable values for the content of free protein SH groups and protein-bound glutathione, compared with the findings obtained in tumor cells during hypoxia (see the table). The use of N-ethylmaleimide had an impact on the metabolism of tumor cells, including the functioning of mitochondria, a change in the content of Ca^{2+} ions and carbonylated proteins, which was accompanied by activation of apoptotic death under hypoxia.

The results of the study confirm the role of the cellular redox status and oxidative modification of proteins in ensuring the functioning of tumor cells, including their mitochondria, at a reduced oxygen tension.

Table

The effect of N-ethylmaleimide and 1,4-dithioerythritol on the implementation of apoptosis, parameters of the glutathione system, and the content of the hydroxyl radical and carbonyl derivatives of proteins in P19 tumor cells under hypoxia, *Me* (Q_1-Q_3)

Studied parameters	Conditions of culturing P19 cells		
	Hypoxia	Hypoxia + NEM	Hypoxia + DTE
Annexin V-FITC+, %	10.75 (4.50–10.90)	98.05 (97.70–98.40) #	5.50 (4.70–6.70)
CD95, u.	1.0 (0.9–1.1)	15.5 (12.8–15.7) #	0.9 (0.8–1.2)
CD120, u.	1.4 (1.3–1.5)	4.1(4.0–4.5) #	1.1 (1.0–1.2)
Cells with reduced ΔΨm, %	10.4 (10.4–10.6)	91.2 (90.8–92.0) #	2.0 (2.0–2.1) #
Hydroxyl radical concentration, nmol/mg of protein	27.21 (23.56–29.93)	29.94 (29.42–32.20)	10.70 (8.93–11.32) #
Intracellular concentration of Ca^{2+}, u.	10.24 (10.10–10.36)	30.23 (29.47–30.39) #	9.48 (9.43–9.49) #
GSH, nmol/mg protein	4.47 (4.40–4.58)	1.30 (0.67–1.88) #	6.00 (5.97–6.32) #
GSSG, nmol/mg protein	0.43 (0.39–0.45)	0.69 (0.50–0.72) #	0.72 (0.34–0.81)
GSH/GSSG	10.19 (9.88–11.35)	1.89 (0.93–4.22) #	8.37 (7.93–17.27)
Protein-bound glutathione, nmol/mg protein	2.08 (1.95–2.19)	2.96 (2.08–3.26)	1.48 (1.43–1.51) #
Protein SH groups, nmol/mg of protein	8.76 (7.83–10.55)	9.62 (9.40–10.28)	12.98 (12.75–13.36) #
Protein carbonyl derivatives, nmol/mg of protein	10.17 (8.92–10.39)	14.34 (14.27–17.63) #	7.54 (6.64–8.32)

Note. ROS – reactive oxygen species, GSH – reduced glutathione, GSSG – oxidized glutathione, NEM – N-ethylmaleimide, DTE – 1,4-dithioerythritol; # – significant differences ($p < 0.05$) compared with the hypoxia group

CONCLUSION

Changes in the redox status of the glutathione system as well as glutathionylation and carbonylation of proteins affect the metabolism of the tumor cell. The effect of 1,4-dithioerythritol promotes the formation of additional mechanisms to escape cell death, and the use of N-ethylmaleimide is accompanied by the activation of apoptosis of P19 tumor cells. Taking into account the leading role of the redox cell homeostasis in triggering apoptosis, our study confirms the need to study the mechanisms of cell death and development of resistance of tumor cells to antitumor drugs, the effects of which are based on a change in the cellular redox status.

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Screening of depression with an assessment of the socioeconomic status of patients in the primary care network in the large industrial city of Eastern Siberia

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ABSTRACT

The aim was to compare the relationship between the severity of depression symptoms among the unorganized population of Krasnoyarsk in 2006 and 2012 with respect to socioeconomic and demographic factors; and to compare their prevalence for the analyzed period.

Materials and methods. Two sample groups were selected from the unorganized population that resided permanently in the territory of Krasnoyarsk in 2006 and 2012. Evaluation of the severity of depression in both cases was carried out according to the Hospital Anxiety and Depression Scale, Depression subscale (HADS-D).

Results. In both sample groups, the frequency of depression was associated with age. In 2012, social and economic factors of depression were revealed: lack of higher education, widowhood, unemployment and family poverty. A significant decrease in the frequency of increased (39.1% versus 16.4%) and clinical depression (14.6% versus 4.5%) was found for the period from 2006 to 2012.

Conclusions. In 2012, the frequency of the above-normal depression level according to HADS-D in working age population was largely determined by the influence of socioeconomic factors. A decrease in the frequency of increased and clinical levels of depression among the adult population of Krasnoyarsk over the period from 2006 to 2012 was established.

Key words: prevalence of depression, prevalence of socioeconomic factors, relationship between depression and socioeconomic factors, gender differences in depression.

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Скрининг депрессии с оценкой социально-экономического статуса пациентов в первичной медицинской сети в крупном промышленном центре Восточной Сибири

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РЕЗЮМЕ

Цель. Оценить взаимосвязь выраженности симптомов депрессии по Госпитальной шкале тревоги и депрессии HADS (The Hospital Anxiety and Depression Scale; подшкала HADS(d)) среди неорганизованного населения г. Красноярска в 2006 и 2012 гг. с социально-экономическими и демографическими факторами, а также сопоставить их распространенности за анализируемый период.

Материалы и методы. Обследованы две выборки, сформированные из неорганизованного населения, постоянно проживавшего на территории г. Красноярска в 2006 и 2012 гг. Оценка выраженности симптомов депрессии в обоих случаях проводилась согласно HADS(d).

Результаты. В обеих выборках отмечена связь частоты симптомов депрессии с возрастом. В 2012 г. выявлены социальные и экономические факторы депрессии: отсутствие высшего образования, вдовство, безработица и бедность семьи. Установлено значимое снижение частоты повышенной (39,1 против 16,4%) и клинической депрессии (14,6 против 4,5%) за период с 2006 по 2012 г.

Заключение. В 2012 г. частота уровня депрессии «выше нормы» по HADS(d) в трудоспособном возрасте во многом обусловлена влиянием социально-экономических факторов. Установлено снижение частоты повышенного и клинического уровня депрессии по HADS(d) среди взрослого населения г. Красноярска за период с 2006 по 2012 г.

Ключевые слова: распространенность депрессии, распространенность социально-экономических факторов, взаимосвязь депрессии и социально-экономических факторов, гендерные различия депрессии.

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

Источник финансирования. Авторы заявляют об отсутствии финансирования.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследования «Профилактика и лечение артериальной гипертонии», «ЭССЕ-РФ 2012» в г. Красноярске были одобрены локальным этическим комитетом (ЛЭК) КрасГМУ (протокол № 23 от 02.04.2006), независимыми этическим комитетом ФГБУ «Национальный медицинский исследовательский центр профилактической медицины» и ЛЭК КрасГМУ (протокол № 12 от 08.10.2012). Разрешение на обработку данных исследования авторами статьи получено ЛЭК ГМУ (протокол № 66 от 15.12.2015).

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INTRODUCTION

In recent years, the problem of growing mood disorders in working age has attracted increasing attention from domestic and foreign researchers. The World Health Organization estimates that by 2020, after cardiovascular disease (CVD), depression will be the second major cause of work disability. Meanwhile, in Russia, there is still a significant variation of data on the prevalence of depression in the general medical care network, which is explained by low screening of depression symptoms at the outpatient stage as well as by the lack of a unified method of its diagnosis [1].

Academician A.B. Smulevich developed a technique to detect anxiety and depressive disorders using psychometric scales where a preference was given to subjective questionnaires. Their completion did not require involvement of a psychiatrist or any special skills for data interpretation by general practitioners [2]. Numerous studies have confirmed validity of the Hospital Anxiety and Depression scale (HADS) used in our research for diagnosing depression in the general medical care network and in the general population. Besides, this questionnaire is simple and requires little time for the patient to fill it in [1, 3, 4].

Currently, depressive disorders play a key role in development of cardiovascular diseases (CVD). Depression is considered to be a bridge between social factors such as income level, family material security, etc. and biological risk factors (RF) [5]. It is well known that low social status is associated with an unfavorable behavior profile (smoking and alcohol abuse), which is triggered by stress and depression [6]. At the same time, there is no consensus on how social factors influence the course of depression in people of working age. A number of researchers believe that a lower risk of depression among working individuals of older age is primarily related to their somatic condition. However, there exists a different point of view claiming that employment is the major factor in protection from depression [6, 7]. In the study by O.V. Tsygankova, the absence of family, low income, age and unemployment demonstrated a strong correlation with the high frequency of subdepression in patients with coronary artery disease (CAD) [8]. According to A.V. Orlov, high occurrence of depression among the adult population of St.-Petersburg is primarily related to low income. In addition, other studies have shown that higher levels of depression are associated with low levels of education rather than with family well-being [5, 9]. In the study by E.V. Lebedeva (2018), social adaptation disorders (income management, family problems) showed a significant correlation with affective disorders among patients with CAD [10].

The only major study on the prevalence of depression was conducted in the city of Krasnoyarsk, which indicated the link between depression and hypertension. Nevertheless, no studies have been done on the correlation between depression and socioeconomic factors. Hence, our work is important and of scientific value [11, 12].

The aim of the study was to assess the correlation between depression symptoms and socioeconomic and demographic factors, as well as to estimate the occurrence of depression symptoms in two independent sample groups formed from the unorganized population of Krasnoyarsk in 2006 and 2012 using psychometric testing (HADS, Depression subscale).

MATERIALS AND METHODS

The work provides analysis of two independent studies. The first study was conducted in 2006 within the framework of the regional targeted program "Prevention and treatment of hypertension". The other one was conducted in 2012 during the multi-center study "Epidemiology of Cardiovascular Diseases in Regions of the Russian Federation – 2012 (ESSE-RF epidemiological study)". The latter is the latest epidemiological research which studied the frequency of CVD risk factors, including depression symptoms among the adult population. The coordinators of the study in Krasnoyarsk, Yu.I Greenstein and M.M. Petrova, focused on the analysis of the traditional risk factors. Attempts to estimate the risk factors and depression have not been made yet [12]. S.A. Shalnova's analysis provides psychometric data for 10 regions participating in the study, similar data for Krasnoyarsk (frequency of increased-level / clinical depression according to HADS-D, gender aspects of depression) are not available [1]. Moreover, no similar studies of random samples were performed in Krasnoyarsk after 2012. The obtained data can be used to develop measures aimed at timely screening and preventing depression in the general medical care network. In both cases, random samples were formed using the Kish selection grid, taking into account the clustering principles and age and gender representation (25–64 years old) [12]. In 2006, 322 people were included in the study in 10 clinics in Krasnoyarsk. The sizes of the representative samples were determined on the basis of the method proposed by V.I. Paniotto (2003), according to which for a total of more than 100 thousand people 400 should be screened [13]. 322 people agreed to participate in the survey – 105 men (32.6%) and 217 women (67.4%), the response to the study was 80.2%. In 2012, 1,123 patients from 4 clinics were examined; the response to the study was 80%. Correct data according to HADS-D were obtained from 1,120 respondents: 408 men (36.4%) and 712 women (63.6%).

The sociodemographic factors assessed in 2006 were age, absence of higher education and disability. In 2012, the analyzed factors were absence of higher education; absence of family; widowhood;

unemployment, and disability. Comparative characteristic of the frequency of the studied parameters in 2006 and 2012 is shown in Table 1.

Table 1

Comparative characteristic of the frequency of the studied factors in 2006 and 2012

Factor	Number and frequency of patients in 2006 (n = 322)	Number and frequency of patients in 2012 (n = 1 120)	p
Female Male	217 (67.4) 105 (32.6)	714 (63.6) 409 (36.4)	0.208
Age: ≥ 45 years; < 45 years	 194 (60.2) 128 (39.8)	 558 (49.8) 562 (50.2)	 <0.001*
Higher education: – yes; – no	 93 (29.2) 226 (70.8)	 602 (53.8) 518 (46.2)	 <0.001*
Disability: – yes; – no	 24 (7.5) 298 (92.5)	 39 (3.5) 1 081 (96.5)	 0.003*

Note. In bold – significant differences between the groups (according to the Mann–Whitney U test for the average level of depression and according to the χ^2 test for other indicators). * level of education was determined in 319 patients: 105 men and 214 women.

The manifestation of depression symptoms was assessed according to the depression subscale of HADS, the reliability, sensitivity and specificity of which in Russia were determined during the study in the COMPASS program. It has been proven that by using this technique, taking into account optimal points of separation, the risk of missing depression is low. When interpreting the results, the total indicator for "Depression (D)" subscale was taken into account: 0–7 points – absence of depression; 8–10 points – subclinical depression; 11 points and more – clinical depression; 8 + – increased depression level – total indicator of subclinical and clinical depression [3, 5]. The data were statistically processed by means of SPSS version 23 (USA) and Microsoft Excel (2010) spreadsheets. The study applied non-parametric criteria – the Mann – Whitney U test for paired comparisons and the Kruskal – Wallis test for multiple comparisons. Quantitative data are presented as the median (Me) with lower (Q_{25}) and upper (Q_{75}) percentiles; qualitative data are presented as relative frequency (%). The qualitative comparison was performed using Pearson's chi-squared test criterion ($\chi 2$), taking into account the degrees of freedom (df). The critical level of statistical significance in the null hypothesis tests was assumed to be 0.05 or less. The article discusses only statistically significant relationships.

RESULTS

The results of the assessment of depression symptoms in both studies are presented in Tables 2 and 3. A significant decrease in the proportion of individuals with increased levels of depression (χ^2 = 76.4, df = 1, p < 0.001) and clinical depression (χ^2 = 40.9, df = 1, p < 0.001) was found. The median of the average depression level in 2006 was significantly higher than in 2012 (p < 0.001).

In 2006, men and women were statistically comparable in both the level (p = 0.722) and frequency of increased depression ($\chi 2$ = 0.2, df = 1, p = 0.641) and clinical depression ($\chi 2$ = 0.1, df = 1, p = 0.820). In 2012, among women, there were slight trends towards higher frequency of increased depression level ($\chi 2$ = 3.4, df = 1, p = 0.065) and clinical depression ($\chi 2$ = 0.44, df = 1,

Table 2

The correlation between depression and gender, level of education, age, senior management position, and disability in 2006

Factor	Average level of depression, Me (Q_{25} – Q_{75})	The number and frequency of patients with different levels of depression (n = 322: 105 men and 217 women)	
		HADS ≥ 8, n (%)	HADS ≥ 11, n (%)
Total sample Men Women	6.0 (3.75 – 10.0) 7.0 (3.5 – 10.0) 6.0 (3.5 – 10)	126 out of 322 (39.1) 43 out of 105 (41.0) 83 out of 217 (38.2)	47 out of 322 (14.6) 16 out of 105 (15.2) 31 out of 217 (14.3)
Higher education:* – yes; – no	 6.0 (3.0 – 9.0) 7.0 (4.0 – 10.0)	 32 out of 93 (34.4) 93 out of 226 (41.2)	 12 out of 93 (12.9) 34 out of 226 (15.0)
Age: ≥ 45 years; < 45 years	 7.0 (5.0 – 10.0) 5.0 (2.0 – 8.0)	 90 out of 194 (46.4) 36 out of 128 (28.1)	 37 out of 194 (19.1) 10 out of 128 (7.8)
Senior management position: – yes; – no	 6.0 (3.75 – 9.0) 7.0 (3.25 – 10.0)	 25 out of 66 (37.9) 101 out of 256 (39.5)	 9 out of 66 (13.6) 38 out of 256 (14.8)
Disability: – yes; – no	 8.0 (5.3 – 11.5) 6.0 (3.0 – 9.0)	 13 out of 24 (54.2) 113 out of 298 (37.9)	 6 out of 24 (25.0) 41 out of 298 (13.8)

Table 3

The correlation between depression and gender, level of education, age, marital status, and employment in 2012

Factor	Average level of depression. *Me* (Q_{25} – Q_{75})	The number and frequency of patients with different levels of depression (*n* = 1,120: 408 men and 712 women)	
		HADS ≥ 8, *n* (%)	HADS ≥ 11, *n* (%)
Total sample	4.0 (2.0 – 6.0)	184 out of 1,120 (16.4)	50 out of 1,120 (4.5)
Men	3.0 (1.25 – 6.0)	56 out of 408 (13.7)	16 out of 408 (3.9)
Women	4.0 (2.0 – 6.75)	128 out of 712 (18.0)	34 out of 712 (4.8)
Higher education:			
– yes;	3.0 (1.0 – 6.0)	83 out of 602 (13.8)	24 out of 602 (4.0)
– no	4.0 (2.0 – 7.0)	101 out of 518 (19.5)	26 out of 518 (5.0)
Age:			
≥ 45 years;	4.0 (2.0 – 6.0)	102 out of 558 (18.3)	33 out of 558 (5.9)
< 45 years	3.0 (2.0 – 6.0)	82 out of 562 (14.6)	17 out of 562 (3.0)
Family:			
– yes;	4.0 (1.8 – 6.0)	148 out of 918 (16.1)	42 out of 918 (4.6)
– no	4.0 (2.0 – 7.0)	36 out of 202 (17.8)	8 out of 202 (4.0)
Widowhood:			
– yes;	5.0 (2.0 – 8.0)	23 out of 86 (26.7)	5 out of 86 (5.8)
– no	4.0 (2.0 – 6.0)	160 out of 1,032 (15.5)	45 out of 1,032 (4.4)
Disability:			
– yes;	5.0 (1.0 – 7.0)	9 out of 39 (23.1)	1 out of 39 (2.6)
– no	4.0 (2.0 – 6.0)	175 out of 1,081 (16.2)	49 out of 1,081 (4.5)
Unemployment			
– yes;	5.0 (1.5 – 7.0)	12 out of 57 (21.1)	7 out of 57 (12.3)
– no	4.0 (2.0 – 6.0)	172 out of 1,063 (16.2)	43 out of 1,063 (4.0)
Family poverty			
– yes;	4.0 (2.0 – 6.0)	33 out of 101 (32.7)	12 out of 101 (11.9)
– no	5.0 (2.5 – 8.0)	151 out of 1,019 (14.8)	38 out of 1,019 (3.7)

p = 0.506), while men had lower levels of depression (p = 0.002). In 2006, depression showed no significant link with socioeconomic factors. In 2012, the incidence of depression symptoms had a pronounced dependence on risk factors. Therefore, in 2006, absence of higher education did not affect the frequency of increased depression level ($\chi2$ = 1.3, df = 1, p = 0.262), but in 2012, this factor was associated with a higher frequency of increased depression level ($\chi2$ = 6.6, df = 1, p = 0.010). In 2012, widowhood was associated with higher incidence of increased depression level ($\chi2$ = 6.6, df = 1, p = 0.010) and unemployment – with a three-fold increase in the likelihood of clinical depression development ($\chi2$ = 6.8, df = 1, p = 0.009 with Yates's correction for continuity). In families with low material well-being, increased and clinical depression levels were significantly more common than in families with better material security ($\chi2$ = 21.3, df = 1, p < 0.001 and $\chi2$ = 14.3, df = 1, p < 0.001, respectively). To analyze the relationship between depression and age, individuals were divided into 2 groups: 25 to 44 years old and 45 to 64 years old. In 2006, the analysis showed predominance of persons with increased ($\chi2$ = 10.8, df = 1, p = 0.001) and clinical depression ($\chi2$ = 7.8, df = 1, p = 0.005) in a more senior age group (≥45 years). In 2012, the association of age with the incidence of depression symptoms was weaker in both strength and statistical significance and achieved significant differences between groups only in regards to clinical depression ($\chi2$ = 5.5, df = 1, p = 0.019).

DISCUSSION

The obtained data concerning the frequency of depression symptoms according to HADS-D among the adult population of Krasnoyarsk in 2006 correspond with the data of S.Yu. Shtarik, showing that depressive disorders were identified in 34.7% of respondents aged 19–64 years in 2004–2008. At the same time, it is impossible to clearly compare the results of both studies due to differences in methods of psychometric testing and the age of the subjects. According to S.A. Shalnova, among 10 Russian regions participating in the ESSE-RF epidemiological study, the increased level of depression according to HADS-D was registered among 25.6% of adult population of Russia, ⅓ of the value (8.8%) being the clinical level. In 2012, the population of Krasnoyarsk was comparable in the frequency of increased and clinical depression with Kemerovsky region (16.7% and 5.0%) and St.-Petersburg (18.9% and 5.7%) [1, 11]. Our results are slightly different from the data of Russian and foreign authors, according to which symptoms of depres-

sion among women are 2–4 times more common (especially in the postmenopausal period) compared to men. In the HAPIEE study, in the sample of 2,151 respondents aged 45–64 years, the frequency of depression among women reached 44%, and among men – 23% according to the Center for Epidemiologic Studies Depression (CES-D) scale. In the work by O.V. Tsygankova, among 245 patients with CAD aged 35–65years, the probability of detection of subdepression using the Zung Self-Rating Depression Scale in women was 41.2% versus 24.0% in men [7, 8, 14]. At the same time, in Krasnoyarsk, groups of patients with depressive disorders in 2004–2008 were comparable in terms of gender composition [11]. It also should be noted that in the "ESSE-RF" study among 10 regions of Russia, men and women differed slightly in the frequency of depression symptoms: 20.6% of women and 20.0% of men with HADS-D ≥ 8. In the work by F.N. Jacka (2011), which studied 2,957 Norwegians aged 46–49 years, the above-normal depression level according to HADS was registered among 9.6% of men and 7.6% of women [1, 15]. The study confirms that low education level and family poverty are factors associated with low social status and high prevalence of depression [1, 11]. In 2012, the link between depression symptoms prevalence and family poverty was stronger and more significant than with low level of education. In 2004–2008, in Krasnoyarsk, patients with depressive disorders according to the Beck Depression Inventory had lower level of education [11]. In the study by A.V. Orlov, among 1,600 residents of St.-Petersburg aged 25–64 years, regression analysis with gender and biological risk factor correction did not confirm a correlation between HADS depression symptoms and low level of education [5]. Loneliness and death of loved ones are predominant risk factors of depression [6]. According to our data, the frequency of depression was maximum in the older age group of 55–64 years old (26.7%). In 2006, the frequency of depression symptoms did not depend on the level of employment. Unemployment was associated with a greater frequency of clinical depression in 2012. At the same time, as presented in the work by O.V. Tsygankova, subsindromal manifestations of depression in women show a stronger connection with unemployment than with age and absence of family [8]. It is obvious that there is a current trend towards weakening of the correlation between age and frequency of depression. According to V.V. Gafarov, the age of 45 years old and more does not affect the prevalence of depression [16]. Meanwhile, in both studies individuals with clinical depression according to the HADS-D scale were most likely to be in the older age group. A similar increase in the gradient of depression symptoms with the increase in age was noted in S.A. Shalnova's study. However, in the HAPIEE study among residents of Novosibirsk, this association turned out to be insignificant [1, 7, 16].

The results of the study are significantly limited by a number of factors: differences in the age of the sample groups; differences in the number of people with disabilities in both groups (which can be explained by their natural death over a period of 6 years); differences in the number of people with higher education (29.2% versus 46.3%, probably due to the population growth in 2012). At the same time, disability and low education level in 2006 did not affect the variability of depression symptoms. Hence, some aspects of our study should be investigated further.

CONCLUSION

Nowadays, the effect of socioeconomic factors on the variability of depression symptoms has increased. If in 2006 such correlations were not observed, in 2012 we were able to identify the social categories of individuals that are most susceptible to depression. In the period from 2006 to 2012, a decrease in the increased and clinical depression levels according to HADS-D was observed among the population of Krasnoyarsk.

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Growth and characterization of a tissue-engineered construct from human coronary artery smooth muscle cells

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ABSTRACT

Aim. To optimize a bioengineered I-Wire platform to grow tissue-engineered constructs (TCs) derived from coronary artery smooth muscle cells and characterize the mechano-elastic properties of the grown TCs.

Materials and methods. A fibrinogen-based cell mixture was pipetted in a casting mold having two parallel titanium anchoring wires inserted in the grooves on opposite ends of the mold to support the TC. The casting mold was 3 mm in depth, 2 mm in width and 12 mm in length. To measure TC deformation, a flexible probe with a diameter of 365 mcm and a length of 42 mm was utilized. The deflection of the probe tip at various tensile forces applied to the TC was recorded using an inverted microscope optical recording system. The elasticity modulus was calculated based on a stretch-stress diagram reconstructed for each TC. The mechano-elastic properties of control TCs and TCs under the influence of isoproterenol (Iso), acetylcholine (ACh), blebbistatin (Bb), and cytochalasin D (Cyto-D) were evaluated. Immunohistochemical staining of smooth muscle α-actin, desmin and the cell nucleus was implemented for the structural characterization of the TCs.

Results. The TCs formed on day 5–6 of incubation. Subsequent measurements during the following 7 days did not reveal significant changes in elasticity. Values of the elastic modulus were 7.4 ± 1.5 kPa on the first day, 7.9 ± 1.4 kPa on the third day, and 7.8 ± 1.9 kPa on the seventh day of culturing after TC formation. Changes in the mechano-elastic properties of the TCs in response to the subsequent application of Bb and Cyto-D had a two-phase pattern, indicating a possibility of determining active and passive elements of the TC elasticity. The application of 1 μM of Iso led to an increase in the value of the elastic modulus from 7.9 ± 1.5 kPa to 10.2 ± 2.1 kPa ($p < 0.05$, $n = 6$). ACh did not cause a significant change in elasticity.

Conclusions. The system allows quantification of the mechano-elastic properties of TCs in response to pharmacological stimuli and can be useful to model pathological changes in vascular smooth muscle cells.

Key words: tissue engineering, vascular smooth muscle cells, smooth muscle tissue construct.

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

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Выращивание и характеризация тканеинженерной конструкции из гладкомышечных клеток коронарной артерии сердца человека

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РЕЗЮМЕ

Цель. Оптимизировать биоинженерную платформу I-Wire для выращивания тканеинженерных конструкций (ТК) из гладкомышечных клеток (ГМК) артериальных сосудов и охарактеризовать механо-эластические свойства полученных ТК.

Материалы и методы. Клеточную смесь на основе фибрина засевали в канал матрицы из полидиметилоксана с вставками из титановой проволоки на противоположных концах канала для горизонтальной поддержки конструкции. Размеры канала: глубина 3 мм, ширина 2 мм и длина 12 мм. Для измерения деформации ТК использовали гибкий зонд диаметром 365 мкм и длиной 42 мм. Отклонение кончика зонда при различной силе растяжения, приложенной к ТК, регистрировали с помощью системы оптической регистрации на основе инвертированного микроскопа. Модуль упругости вычисляли на основе диаграмм растяжения ТК. Были оценены механо-эластические свойства конструкций в контроле и под действием изопротеренола (Изо), ацетилхолина (Ацх), блеббистатина (Бб) и цитохалазина Д (Цито-Д). Для структурной характеризации конструкций использовали метод иммуногистохимического окрашивания конструкций на α-актин гладких мышц, десмин и ядра клеток.

Результаты. Формирование конструкций происходило на 5–6-й день инкубации. Последующие измерения в течении 7 дней не выявили значительных изменений эластичности. Значения величины модуля упругости конструкций составили 7,4 ± 1,5 кПа в первый день после их формирования, 7,9 ± 1,4 кПа – на 3-й и 7,8 ± 1,9 кПа – на 7-й день культивирования. Изменения механо-эластических свойств ТК в ответ на последовательное применение Бб и Цито-Д имели двухфазный характер, что демонстрирует возможность выделения активного и пассивного элементов эластичности гладкомышечных конструкций. Добавление 1 мкМоль Изо приводило к увеличению значения величины модуля упругости с 7,9 ± 1,5 кПа до 10,2 ± 2,1 кПа ($p < 0,05$, $n = 6$). Добавление Ацх не вызывало значимого изменения эластичности.

Заключение. Представленная система позволяет количественно оценивать механо-эластические свойства ТК в ответ на фармакологическое воздействие и может быть полезна в моделировании патологических изменений в ГМК сосудов.

Ключевые слова: тканевая инженерия, васкулярные гладкомышечные клетки, гладкомышечная тканеинженерная конструкция.

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

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INTRODUCTION

Vascular smooth muscle cells (VSMCs) are the main type of cells in the vascular system that determine vascular tone, as well as peripheral and arterial blood pressure. A feature of VSMCs is their incomplete differentiation and, as a consequence, high plasticity associated with changes between contractile and secretory phenotypes in response to biochemical or mechanical stimulation [1]. It has been shown that a change in the VSMC phenotype is involved in such pathologies as atherosclerosis, aortic aneurysm, and arterial hypertension [2–4].

At present, in most experimental studies of the mechanisms of vascular diseases *in vitro*, traditional two-dimensional cultures of VSMCs are utilized. Such experimental models represent a non-physiological environment, which makes it impossible to maintain the original phenotype of cells and complicates interpretation of data. The modern development of tissue engineering methods allows us to reproduce quite well the basic physiological properties of various types of tissues [5–7]. The advantages of engineered three-dimensional tissue constructs (TCs) are the appropriate mechanical environment, ensuring the stability of the response to external stimulation over a long period of time, the relative ease of genetic manipulations, and the unique potential for studying the biophysical characteristics of tissues *in vitro* [8].

Thus, the development of an adequate *in vitro* model both for studying the mechanisms of vascular diseases and for testing drugs is of paramount importance. This work is aimed at optimizing the novel I-Wire bioengineering platform developed at Vanderbilt University [9, 10], growing TCs using arterial VSMCs and characterizing the mechano-elastic properties of the artificial tissue constructs.

MATERIALS AND METHODS

Preparation of PDMS mold and cell mixture. In this work, a polydimethylsiloxane (PDMS) mold with a horizontal support (titanium wire with a diameter of 0.25 mm and a length of 12 mm, Sigma-Aldrich, USA) for the TC was utilized. To prepare the mold, a template with six cavities was made from monolithic acrylic plastic using a drill with a diameter of 0.79 mm and a numerically controlled milling machine (MicroProtoSystems, USA). Two thin edges of the same material were inserted into each cavity to form channels for the supporting titanium wire.

The cavities were filled with liquid PDMS (SYLGARD ™ 184 kit, DowCorning, USA) mixed with a hardener in a ratio of 10:1 and degassed. Then, the assembled structure was placed in an oven for 6 hours at 65 °C for polymerization. The final PDMS matrix had a channel with a depth of 3 mm, a width of 2 mm and a length of 12 mm, as well as two grooves to accommodate the anchor wire. Next, each PDMS mold was transferred to the well of a 6-well plate and glued to the bottom using liquid PDMS. To increase the hydrophobicity, the channels of the PDMS devices were treated with 0.2% Pluronic® F-127 solution (Sigma-Aldrich, USA) and then washed with deionized water. Then the 6-well plate with molds was sterilized by UV radiation for 30 minutes.

To grow TCs using commercial VSMCs (HCASMC, ThermoFisher, USA), a fibrin-based cell matrix was prepared. It has been shown that fibrin degradation products, which are produced during the maturation of the construct, promote the proliferation of VSMCs and stimulate the formation of the extracellular matrix [11] . In preliminary experiments for optimizing the conditions for the formation of the construct, a cell mixture of fibroblasts and VSMCs in a proportion of 1:10 was used. When only VSMCs were utilized, the final concentration of cells and other components of the medium was the same.

Cardiac fibroblasts (NHCF-V, Lonza, USA) and VSMCs were collected from T-175 flasks (ThermoFisher Scientific, USA) using TrypLE™ Express Enzyme (Thermo Fisher Scientific, USA). The total cell density was adjusted to 10^6, 2×10^6, or 4×10^6 cells/ml. Thereafter, the cells were mixed with fibrinogen (100 µl, 20 mg/ml, Sigma-Aldrich, USA), aprotinin (33 µg/ml, Sigma-Aldrich, USA), thrombin (10 µl, 100 U/ml, Sigma-Aldrich, USA), and penicillin/streptomycin (1%, Gibco, USA). In addition, Matrigel™ (100 µl, BD Biosciences, USA) was applied depending on experimental conditions. After dilution, the final cell density was 0.5×10^6, 10^6, or 2×10^6 cells/ml. Next, the cell mixture was pipetted into the channel of each PDMS device and cells were incubated at 37 °C and 5% of CO_2 for one hour for fibrin polymerization. Later, 3 ml of Gibco™ Medium 231 (ThermoFisher Scientific, USA) was added to each well of the plate. This medium contained aprotinin (33 µg/ml, Sigma-Aldrich, USA), tranexamic acid (400 µmol, Sigma-Aldrich, USA), antibiotics (1%, Gibco, USA) and either growth supplement (Gibco™ Smooth Muscle Growth Supplement, SMGS) (Thermo Fisher Scientific, USA) or differentiation supplement (Gibco ™ Smooth Muscle Differentiation Supplement, SMDS) (ThermoFisher Scientific, USA) as needed.

The medium was replaced every second day. After the formation of TCs, their mechano-elastic properties were measured using an optical recording system based on an inverted microscope (Fig. 1). The matured smooth muscle fibers were 350–450 μm in diameter and 7 mm long (Fig. 2, *a*).

Setup to measure mechano-elastic properties in engineered TCs. To measure elasticity, a flexible sensor made of polyetheretherketone tubing (Putnam Plastics, USA) with an outer diameter of 365 μm, an inner diameter of 120 μm, and a length of 42 mm was utilized. The sensor was glued to a console made of organic glass, which, in turn, was attached to a holder plate with two magnets to control the sensor position. The console, holder plate and flexible sensor were mounted by friction mounting to the condenser of an inverted fluorescence microscope (Eclipse Ti, Nikon, USA) equipped with a microcontroller (MS-2000 Flat-Top XYZ Automated Stage, ASI, USA), which allows for precise movement of the platform in two dimensions (Fig. 1). The elasticity of the flexible sensor was calibrated; it demonstrated a linear dependence of the strain on the applied force (Fig. 2, *b*). The optical registration of displacement of the flexible sensor tip was carried out with a digital camera (Zyla sCMOS Camera, Andor Technology, Northern Ireland) at equal intervals of the displacement of the platform with a multiwell plate, so that each displacement corresponded to a certain applied force, the value of which was calculated using a calibration graph.

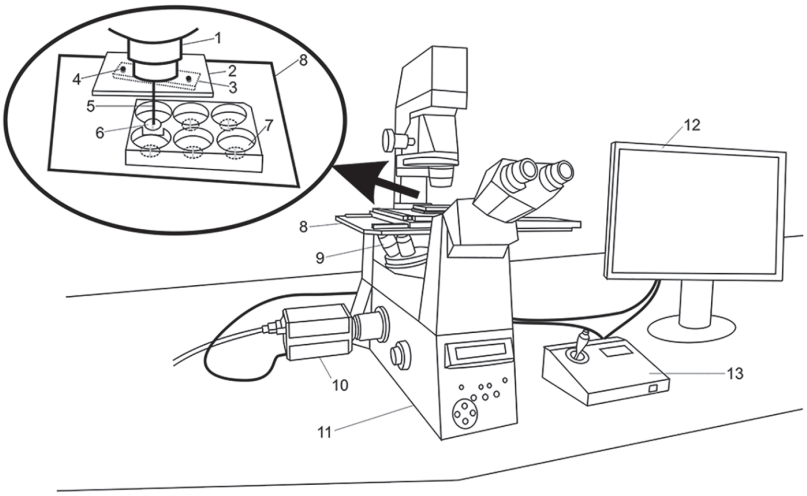


Fig. 1. Schematic diagram of the setup for measuring the mechano-elastic properties of tissue-engineered constructs: *1* – condenser, *2* – plate holder, *3* – console, *4* – magnet, *5* – flexible sensor, *6* – PDMS device with a construct, *7* – well of the multiwell plate, 8 – motorized platform, *9* – lens, *10* – CMOS camera, *11* – inverted microscope, *12* – computer, *13* – microcontroller

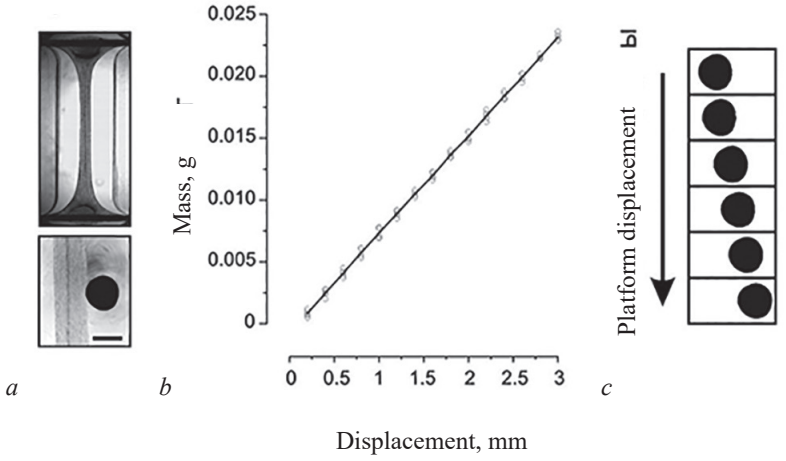


Fig. 2. Calibration graph of elasticity and displacement frames of the flexible sensor tip: *a* – general view of the construct and its central part with the end of the flexible sensor; scale bar – 300 μm, *b* – linear dependence of the change in mass on the offset position of the console with a flexible sensor ($N = 5$, $R = 0.99997$**), *c* – processed binary images of the sensor position during a gradual shift of the motorized platform with the multiwell plate

Measurement protocols and immunohistochemical staining. Two protocols were utilized in this study. The first protocol consisted of a series of sequential measurements of the position of the flexible sensor end when the platform was displaced for every 200 μm before the introduction of substances and after incubation with substances for two hours. According to the second protocol, the registration was carried out at a constant applied force F_t = 0.062 mN for 95 min. During the first 15 min, control registrations were performed. Next, 30 μm of blebbistatin (Bb) and 50 μm of cytochalasin D (Cyto-D) were added after 20 min and 55 min from the start of the protocol, respectively. To assess the ability of the cells to respond to β-adrenergic and cholinergic stimulation, the TC was incubated in standard media containing 1 μm of isoproterenol (Iso) (Sigma-Aldrich, USA) and 5 μM of acetylcholine (ACh) (Sigma-Aldrich, USA) for 60 min. In these experiments, the first protocol was applied.

Bb and Cyto-D were utilized to assess the effect of pharmacological stimuli on the contractile function and elasticity of the extracellular matrix of the TC. Blebbistatin is a reversible inhibitor of ATPase activity of myosin II [12], while cytochalasin D blocks actin polymerization, thereby disrupting the organization of the cytoskeleton filament network, but does not affect the extracellular matrix [13].

For immunohistochemical staining, the TCs were fixed in a paraformaldehyde solution (4%) for 15 min, washed three times with sodium phosphate buffer, then embedded in paraffin blocks and sliced into 5-μm sections. The antigen availability was ensured by incubation of sections at 94 °C in 10 mM sodium citrate solution (pH 6.0) for 10 min. The following antibodies were used in this study: primary murine monoclonal antibodies against smooth muscle cell α-actin (α-SMA) (Sigma-Aldrich, USA), rabbit monoclonal antibodies against desmin (Abcam, USA), secondary antibodies against mice (ThermoFisher, USA) and rabbit (ThermoFisher, USA) conjugated to Alexa (568 and 488 nm), and DAPI for nuclei staining (Fluoromount-G 368/451, SouthernBiotech, USA). Color images were acquired with a Zeiss LSM780 confocal microscope (Zeiss, Germany).

Data analysis and statistics. Image analysis was performed with MATLAB software (MathWorks, USA). The contrast of the image was adjusted to observe only the end of the flexible sensor. Then a binary image was created and the coordinates of the centroid were calculated in each frame (Fig. 2, *c*). Based on the obtained data, the change in the TC length, the value of the force acting along the structure (F_c), and the value of the elastic modulus were calculated. A detailed description of the calculation of the elasticity modulus and a diagram of the geometry of the structure is presented in the supplemental material of a previously published work [9]. Therefore, we provide only a brief description of the calculation procedure.

The elastic modulus was calculated as the ratio of the dependence of strain on stress according to the following equation [14]:

$$E = \frac{L_0 F_c}{A_0 \Delta L},$$

where *F*c is the force acting along the TC, *L*o is the initial length of the TC, *A*o is the cross-sectional area, and Δ*L* is the change in the length of the TC.

Given the position of the TC in space, the formula for calculating the modulus is modified according to [9]:

$$E = \frac{2L_0 F_t \sqrt{(d_s - d_t)^2 + a^2}}{\pi D^2 \Delta L (d_s - d_t)},$$

where F_t – the force exerted by the flexible sensor, d_c – the distance between the initial and current position of the platform, d_t – the distance between the initial and current position of the end of the flexible probe, *a* – half of the length of the TC in a relaxed state, and *D* – the diameter of the TC. The diameter of the TC was calculated based on the average value of three measurements: at the midpoint and at two points at a distance of 1 mm to the right and left of the midpoint.

Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, USA) and OriginLab R2018 (OriginLab Corporation, USA). Statistical comparisons between the control and experimental groups were made with Student's t-test for dependent samples, one-dimensional analysis of variance for repeated measurements (ANOVA) in the case of three or more groups, and non-parametric paired Wilcoxon test. The change in length in response to the applied force, measured as a percentage of the initial length of the preparation, is presented as the mean and standard error of the mean. Differences in the results were considered statistically significant at a significance level of $p < 0.05$. In the process of calibrating a flexible sensor, a correlation analysis was used to assess the linearity of its elastic properties depending on the load.

RESULTS AND DISCUSSION

The main results of preliminary experiments to optimize the protocol revealed that (*a*) the use of a medium with SMDS did not cause the formation of tissue fibers; (*b*) the addition of Matrigel™ accelerated gel condensation and the formation of TCs; (*c*) the VSMC density of 10^6 cells/ml was optimal to enable the rapid growth of the TCs; and (*d*) the addition of 10% fibroblasts did not affect the rate of TC formation.

We utilized fluorescence microscopy to characterize the structure of the TCs. Figure 3 illustrates TC immunohistochemical staining against smooth muscle α-actin, desmin and nuclear DNA with DAPI, as well as hematoxylin and eosin staining. α-Actin constitutes the largest protein fraction in the VSMCs, plays a major role in the contraction via interaction with myosin, and also participates in the formation of the cytoskeleton through its polymerization [15]. Desmin is the main protein that makes up the intermediate filament of the contractile apparatus and is located in both the Z-line of the striated muscle and in the dense bodies of smooth muscle tissue [16]. The immunohistochemical staining data demonstrate the structural homogeneity, as well as uniform and dense distribution of smooth muscle cells in the TCs.

To verify the stability of the mechano-elastic properties of the TCs, control measurements were performed within 7 days after construct formation. The tension diagrams are shown in Fig. 4, A. The linear part of the diagram was utilized to calculate the elastic modulus (Fig. 4, *b*). Substantial TC elongation is observed when a transverse tensile force (F_t) is applied greater than 0.07 mN. Analysis of tensile diagrams did not reveal statistically significant changes between measurements on the 1st, 3rd and 7th days (F (2.10) = 0.225, p = 0.8, n = 6). The value of the elastic modulus was 7.4 ± 1.5 kPa at the beginning of the experiment, 7.9 ± 1.4 kPa on the third day and 7.8 ± 1.9 kPa on the seventh day.

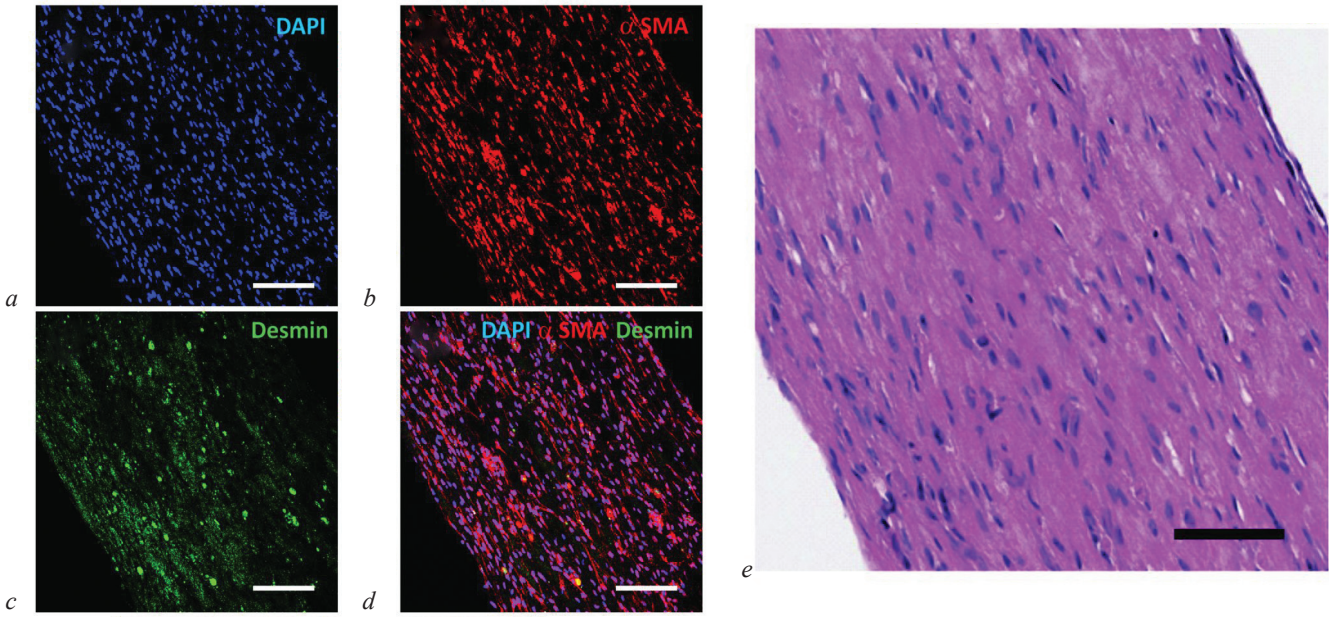


Fig. 3. Immunohistochemical analysis of longitudinal sections of the construct: *a* – DNA staining with DAPI, *b*, *c* – staining for the main markers of the contractile phenotype α-actin (red) and desmin (green), *d* – combined image, *e* – hematoxylin and eosin stain. The scale bar is 100 μm

In the literature, the elasticity data of smooth muscle TCs vary depending on the method of engineering. In particular, elasticity depends on load: static or variable; the composition of the culture medium; the geometry of constructions: ring-shaped [5, 6] or fiber-shaped [7]; the type of matrix: collagen-based, fibrin-based; as well as the origin of the VSMCs and their final density in the cell's mixture [5, 17–20]. The data obtained in individual VSMCs are less variable and are mainly determined by the phenotype of cells [21–23]. Specifically, when measuring elasticity by atomic force microscopy in constructs grown using VSMCs isolated from the thoracic aorta of a monkey (*Macaca fascicularis*) and collagen matrix, elastic modulus

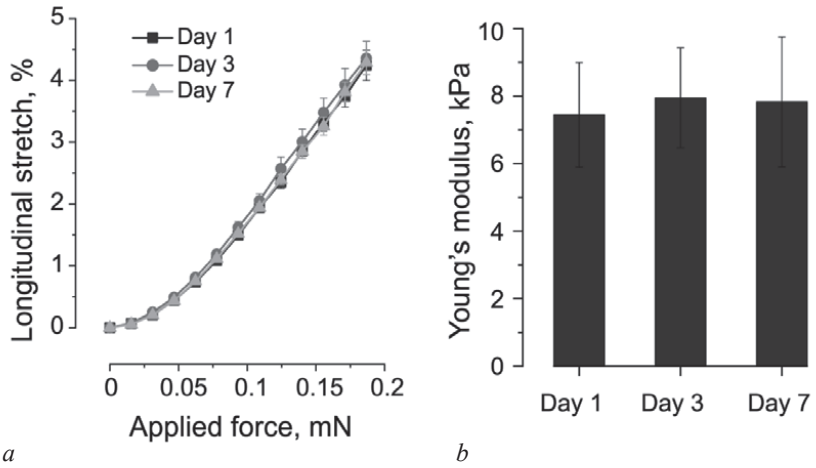


Fig. 4. The stability of the mechanical properties of tissue-engineered constructs: *a* – stress-stretch diagram of the constructs. Repeated measurements at 1st, 4th and 7th days, *b* – calculated elastic modulus (Young's modulus). The data are presented as Mean ± *SEM, N* = 6. There are no statistical differences between the three groups

values were within 13.7 ± 2.4 kPa and 23.3 ± 3.0 kPa [24]. The authors attributed the observed variability to age-related changes in the expression of actin and β1 subunit of integrin [25]. The elasticity assessed with the same method, but in individual VSMCs isolated from the thoracic aorta of healthy young rats (*Wistar-Kyoto*), varies between 5 and 14 kPa [21, 23]. Similar values of the elastic modulus (~13.7 kPa) were demonstrated in individual VSMCs isolated from rat (Sprague – Dawley) skeletal muscle arterioles and cultured for 3–7 days after the first passage [22].

D. Seliktar et al. performed isometric measurements of elasticity using a force sensor in ring-shaped three-dimensional TCs grown in a collagen matrix (2 mg/ml) using rat thoracic aorta VSMCs (10^6 cells/ml) after 8 days of culture, which showed a value of 68 kPa [18]. In another work, wherein the authors compared the elastic properties of the ring-shaped constructs grown with fibrin-based (2 mg/ml) or collagen-based (2 mg/ml) matrixes but with the same type of VSMCs (rat aorta, 10^6 cells/ml), it was shown that after 5 days of culture, collagen-based TCs had significantly greater rigidity (191 kPa) as compared with fibrin-based constructs (19 kPa) [20]. An increase in the concentration of collagen and fibrin in the cell mixture up to 4 mg/ml led to a rise in rigidity to 242 kPa and 28 kPa, respectively.

The elasticity values of smooth muscle TCs attained in our work are close to those measured in individual VSMCs. This could be explained by the use of fibrin in the cell mixture. Fibrin-based matrix is widely used in tissue engineering [26]. Fibrin gel has vasoactive and high adhesive properties and promotes rapid cell adaptation and proliferation in constructs [11], but due to its structural features it has considerable elasticity [27]. It should be noted that because of high plasticity of VSMCs [28, 29], the elasticity of constructs can substantially change during culturing [18, 19]. This can complicate the interpretation of the data, especially in long-term experiments, such as using long-term mechanical stimulation [18]. The application of fibrinolysis inhibitors (aprotinin, tranexamic acid) and low serum supplement (SMDS) in our work contributed to the stability of the elastic properties of the TCs after their maturation (Fig. 4).

Figure 5 depicts the results of a separate experiment to test the mechano-elastic properties of TCs under the influence of Bb and Cyto-D. It can be seen that the addition of 30 μm of Bb causes stretch of the TC from 1.2% to 1.45% (Fig. 5, *a*). Subsequent treatment with Cyto-D at a concentration of 50 μm promotes further relaxation of the TC and an increase in elongation to 1.8%. This correlates with changes in elasticity corresponding to the applied force of 0.062 mN in the tensile diagram of the TC shown in Fig. 5, *b*. It is known that Bb is a selective inhibitor of the ATPase activity of various isoforms of myosin II in striated muscle cells [12] and smooth muscle cells [30], while Cyto-D alters the mechanical properties by depolymerization of the cytoskeleton actin filaments [13]. Accordingly, the addition of Bb inhibits myosin-related contraction, whereas subsequent incubation with Cyto-D destroys the actin-mediated tonic contraction and increases the relaxation of the TC.

Adrenergic and cholinergic stimulations are one of the most important systems involved in the regulation

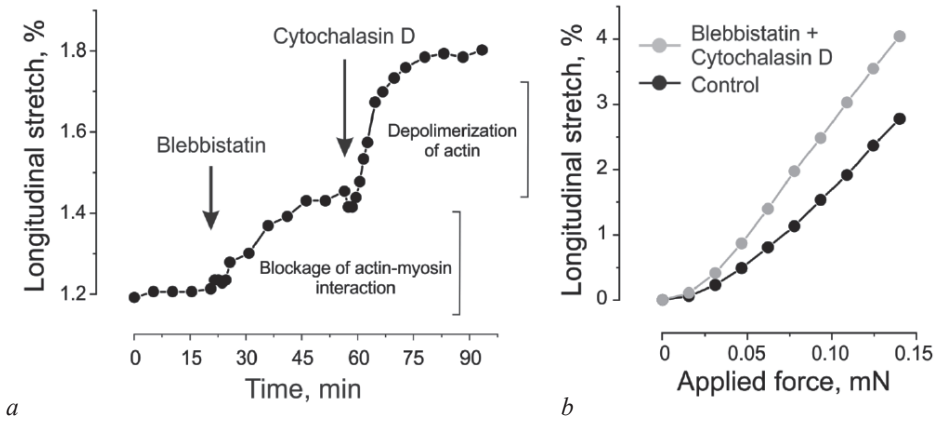


Fig. 5. Effect of blebbistatin and cytochalasin D on the mechano-elastic properties of TCs: *a* – changes in the elasticity of the construct in response to successive treatment by blebbistatin and cytochalasin D at a constant force of 0.062 mN, *b* – stress-stretch diagrams of the constructs at control and after application of 30 μm blebbistatin and 50 μm cytochalasin D

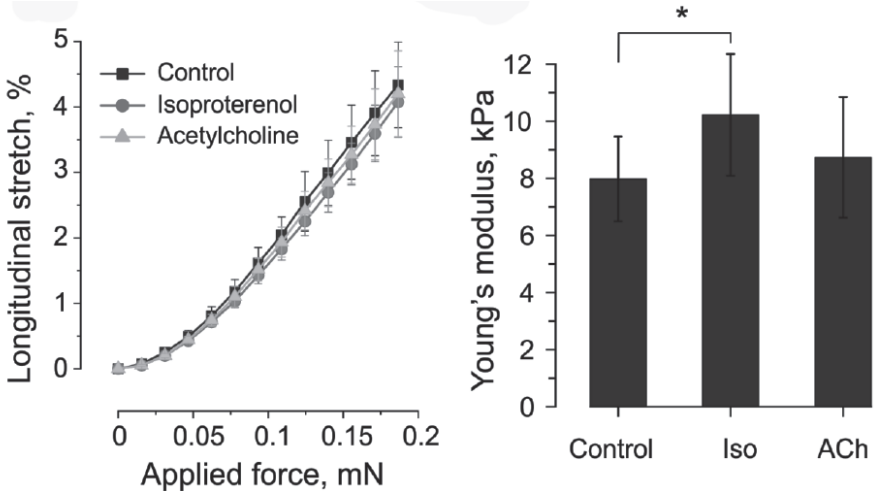
of cardiovascular tone. It is known that β-adrenergic stimulation through activation of adenylate cyclase and an increase in cAMP affects the level of intracellular Ca^{2+} [31, 32]. Several mechanisms causing relaxation were proposed, including hyperpolarization through Ca^{2+} activated potassium channels, reduction of the sensitivity of contractile elements to Ca^{2+} due to phosphorylation of the kinase of myosin light chains, a decrease in the content of cytosolic Ca^{2+} through regulation of Ca^{2+} transporting systems of the sarcoplasmic reticulum and plasmalemma, etc. [33, 34]. On the other hand, VSMCs are in close structural and functional interaction with endothelial cells, which can regulate vascular tone through paracrine interaction or through intercellular channels connecting two types of cells [35]. Therefore, adrenergic regulation of VSMCs contractility mediated by endothelial cells is also possible [35–37].

In our experiments, the incubation of TCs with Iso at a concentration of 1 μm caused a small but significant decrease in elasticity with an increase in the applied tensile strength above 0.05 mN ($p < 0.05$, $n = 6$), as well as a significant increase in the elastic modulus ($p < 0.05$, $n = 6$) from 7.9 ± 1.5 kPa to 10.2 ± 2.1 kPa (Fig. 6). In early studies on the effects of β-adrenergic stimulation on vascular tone and contractility of VSMCs, the experiments were mainly carried out *in vivo* or *in vitro* on isolated preparations of animal vessels, where the relaxing effect is typical [32, 33, 35]. In isolated VSMCs, a double mechanism of regulation of calcium channels was demonstrated. At nanomolar concentrations, Iso increased the L-type calcium channel current, while at micromolar concentrations it had the opposite effect [38]. Since we used only one type of cells (VSMCs) to engineer constructs, the dependence of the response mechanism on the concentration of Iso may underlie the observed decrease in elasticity.

The effect of ACh on VSMCs is mediated by M_3 muscarinic receptors [39]. According to the concept of participation of endothelial cells in the humoral regulation of vascular tone, endothelial cells play a primary role in response to cholinergic stimulation, whereas the adjoining VSMCs are passive recipients of nitric oxide [40]. However, from early works it is known that in the case of an immediate effect of ACh on VSMCs, ACh binds directly to the M_3 receptor, activates phospholipase C and can induce a contractile effect through the inositol triphosphate pathway [41]. In our experiments, the application of ACh caused only a slight decrease in the TC elasticity (Fig. 6, B). The small effect is most likely explained by a homogeneous cell population, i.e., the absence of endothelial cells in the cell mixture during the engineering of the constructs. Future experiments with the addition of a fraction of endothelial cells will allow us to evaluate the role of interactions between various types of cells in both cholinergic and adrenergic regulation of contractility of smooth muscle TCs.

CONCLUSION

In the current work, we for the first time utilized the I-Wire experimental platform to engineer and characterize smooth muscle TCs using VSMCs isolated from the human coronary artery.



a *b*

Fig. 6. Changes in the elasticity of tissue-engineered constructs in response to adrenergic and cholinergic stimulation: *a* – stress-stretch diagrams in the control, under isoproterenol and acetylcholine *b* – change of the modulus of elasticity (Young's modulus) after application of isoproterenol and acetylcholine. The data are presented as *Mean* ± *SEM*. **p* < 0.05, *N* = 6

We showed that a cell density of 10^6 cells/ml is optimal for tissue fiber formation with uniform and dense distribution of cells. The addition of Matrigel™ to the cell mixture accelerates formation of the construct. The combined application of Bb and Cyto-D makes it possible to isolate and evaluate the active and passive elements of elasticity of the TC. Addition of Iso caused an increase in the rigidity of the structures, while incubation with ACh did not have a significant effect on the TC elasticity. Since endothelial cells play an important role in the regulation of vascular smooth muscle tone, in order to reproduce a more complete regulation system in engineered TCs, further experiments should be conducted with co-culture of both VSMCs and endothelial cells, as well as with the use of elastin and collagen to form a stiffer extracellular matrix.

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State of the antioxidant system in mitochondria of skin cells during experimental B16/F10 melanoma growth with chronic neurogenic pain

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ABSTRACT

Aim. To study the state of the antioxidant system in mitochondria of skin cells during B16/F10 melanoma growth in mice with chronic neurogenic pain.

Materials and methods. The study included female C57BL/6 mice (n = 28). Experimental groups included an intact group, a control group – chronic neurogenic pain model, a comparison group – standard subcutaneous transplantation of B16/F10 melanoma, and a main group – transplantation of B16/F10 melanoma 3 weeks after creation of a model of chronic neurogenic pain. Animals were decapitated on day 14 of the B16/F10 melanoma growth, the skin was excised and mitochondria were isolated. Standard ELISA test systems were used to determine the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Bio Source, USA); glutathione peroxidase-4 (GPx 4) (Clod-Clon Corporation, CNDR); glutathione reductase (GR) (Cusabio, CNDR); glutathione S-transferase (G-S-T) (Ivvundiagnostik, FRG); glutathione peroxidase-1 (GPx 1), and superoxide dismutase-2 (SOD-2) (Ab Frontier, South Korea).

Results. Mitochondria of skin cells in controls showed an increase in the levels of GSH by 1.3 times, GPx 1 – by 2.9 times, GPx 4 – by 1.9 times, GR – by 2.8 times, and SOD-2 – by 2.4 times, compared to intact animals. Changes in the comparison group were opposite: GPx 1 decreased by 1.9 times, GPx 4 – by 3.7 times, GR – by 3.9 times, SOD-2 – by 3.8 times, and GSSG rose by 1.36 times compared to intact animals. The growth of melanoma with chronic neurogenic pain caused an increase in the levels of GSH by 1.5 times, GPx 1 – by 3.6 times, G-S-T – by 1.28 times, GPx 4 – by 1.6 times, and SOD-2 – by 1.8 times, compared to intact animals.

Conclusions. The growth of B16/F10 melanoma together with chronic neurogenic pain restructures the antioxidant system of skin mitochondria towards generation of reductive stress under the influence of chronic pain, which can affect the growth and development of experimental melanoma.

Key words: experimental B16/F10 melanoma, chronic neurogenic pain, skin, antioxidant system, mitochondria.

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 animal studies were conducted in compliance with humanity principles set forth in the Directive of the European Union (86/609/EEC) and the Declaration of Helsinki. The study was approved at the session of the Bioethics Committee for Working with Animals of Rostov Research Institute of Oncology (Protocol No. 4 dated 10/08/2018). All participants of the study signed an informed consent to participate in the research.

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Состояние антиоксидантной системы в митохондриях клеток кожи при росте экспериментальной меланомы B16/F10 на фоне хронической нейрогенной боли

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РЕЗЮМЕ

Цель – изучить состояние антиоксидантной системы в митохондриях клеток кожи при росте меланомы B16/F10 у мышей на фоне хронической нейрогенной боли.

Материалы и методы. Работа выполнена на самках мышей линии C57BL/6 (n = 28). Экспериментальные группы: интактная, контрольная – воспроизведение модели хронической нейрогенной боли, группа сравнения – стандартная подкожная перевивка меланомы B16/F10, основная группа – перевивка меланомы B16/F10 через 3 нед после создания модели хронической нейрогенной боли. Животных на 14-е сут роста меланомы B16/F10 декапитировали, иссекали кожу, выделяли митохондрии. Тест-системой для иммуноферментного анализа определяли уровень восстановленного глутатиона (GSH), окисленного глутатиона (GSSG) (Bio Source, США); глутатионпероксидазы-4 (ГПО-4) (Clod-Clon Corporation, CNDR); глутатионредуктазы (ГР) (Cusabio, CNDR); глутатион-S-трансферазы (ГТ) (Ivvundiagnostik, FRG); глутатионпероксидазы-1 (ГПО-1), супероксиддисмутазы-2 (СОД-2) (Ab Frontier, Южная Корея).

Результаты. В митохондриях клеток кожи в контрольной группе установлено повышение содержания GSH в 1,3 раза; ГПО-1 – 2,9; ГПО-4 – 1,9; ГР – 2,8; СОД-2 в 2,4 раза относительно значений у интактных животных. В группе сравнения обнаружили принципиально противоположные изменения: снижение содержания ГПО-1 в 1,9 раза; ГПО-4 – 3,7; ГР – 3,9; СОД-2 в 3,8 раза и повышение уровня GSSG в 1,36 раза по сравнению со значениями у интактных животных. При росте меланомы на фоне хронической нейрогенной боли отмечено увеличение уровня GSH в 1,5 раза; ГПО-1 – 3,6; ГТ – 1,28; ГПО-4 – 1,6 и СОД-2 в 1,8 раза по сравнению со значениями в интактной группе животных.

Заключение. При росте меланомы B16/F10 на фоне хронической нейрогенной боли происходит перестройка антиоксидантной системы митохондрий клеток кожи в сторону реализации «восстановительного стресса» под воздействием хронической боли, что может оказывать влияние на рост и развитие экспериментальной меланомы.

Ключевые слова: экспериментальная меланома B16/F10, хроническая нейрогенная боль, кожа, антиоксидантная система, митохондрии.

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

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

Соответствие принципам этики. Исследование одобрено биоэтическим комитетом по работе с животными Ростовского научно-исследовательского онкологического института (протокол № 4 от 10.08.2018).

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INTRODUCTION

Skin melanoma is characterized by an extremely high degree of malignancy and an exceptionally high potential of lymphatic, hematogenous or lymphohematogenous spread. Traditionally, skin melanoma is considered to have a variable, frequently unpredictable clinical progression, including both cases of spontaneous involution of the primary tumor lesion and early spread of the neoplastic process with favorable prognostic signs available [1]. In recent years,

certain success has been achieved in understanding the etiology of this disease which is associated with the anatomic localization, the extent of exposure to ultraviolet radiation, genetic particularities, and, potentially, other factors, too [1, 2].

Any pathological process undergoes the initial change at the hyperfine level. The borderline of the transition from the norm to pathology requires an in-depth study at the atomic, molecular, and sub-cellular levels [3]. The ability to adapt cellular bioenergetic capacities under the influence of rapidly changing conditions of the environment is compulsory both in the normal cellular function and in the development of tumors [4]. It is mitochondria that are highly sensitive indicators of pathological processes emerging in the body. Being the central metabolic organelle, they perform crucial biochemical functions in synthesizing the main cellular components, including fatty acids, aminoacids, and nucleotides. Cells of many tumors containing completely functional mitochondria increase the speed of glycolysis for maintaining proliferation and survival, thus ensuring a higher flow of substrate for biosynthesis pathways partially performed in mitochondria (metabolism of glucose and lipogenesis, metabolism of aminoacids, and biosynthesis of nucleotides). As a result of the activation of the metabolic flow through mitochondrial pathways, the production of ROS in tumor cells increases, which entails activation of antioxidative response pathways of the cells [4].

The skin is a common target organ for melanoma, and it is a unique and the largest organ/tissue of the body accumulating numerous physiological functions. The skin is a standalone organ and the key interface between the endocrine, nervous and immune systems. The skin is an integral sophisticated tissue system incorporating several layers which are closely connected with one other. In the skin, circulatory, lymphatic and nervous pathways are present that enable it to quickly respond to pain, mechanic, chemical, thermal, and other stimulations by luminal narrowing or dilation with the subsequent change of the blood flow. The vascular tone (lumen of vessels and blood flow speed) of the skin is influenced by the cerebral cortex via numerous vasoconstrictor and vasodilator nerve endings, owing to which the body ability to feel warmth, cold, pressure, tactile sense, and pain is fulfilled [5]. Pain is frequently an accompanying component of a neoplastic process, and it is present in 30 – 50% of cancer patients after the performed anticancer therapy and in 65 – 90% of patients due to disease progression [6]. As a rule, the origin of pain in oncological patients is multi-factorial: it arises from direct and indirect effects of the tumor growth, a side effect of antineoplastic therapy, and concurrent diseases [6]. Experimental oncology moves towards understanding the biological and physiological processes arising in the body in combined concurrent chronic pain and tumor development. In particular, chronic neurogenic pain was reported to stimulate and modify the growth of cutaneous melanotic cancer in the experiment [7]. Therefore, studying antioxidative processes in mitochondria of skin cells under pathological processes accompanied by pain syndrome seems highly relevant. Certainly, pathophysiological processes can be studied by means of experimental models. It is indisputable that development of experimental oncology with an extensive study of pathophysiology of the malignant process using experimental animal models promotes advancement of both practical and theoretical oncology.

The aim of the research was to study the antioxidative system in mitochondria of skin cells in experimental animals with chronic neurogenic pain, tumor development, and the combined effect of these pathological processes.

MATERIALS AND METHODS

The study was performed using female C57BL/6 mice (n = 28) aged 8 weeks weighing initially 21–22 g. The animals were received from Scientific Center of Biomedical Technologies "Andreevka" (Moscow region). The cell culture of murine B16/F10 melanoma metastasizing into the lungs was used. The tumor strain was obtained from N.N. Blokhin National Medical Research Center of Oncology.

The animals were kept under natural lighting mode with free access to water and food. All studies were conducted in compliance with the requirements and conditions stated in the International Guiding Principles for Biomedical Research Involving Animals and the Order of the Russian Ministry of Health No. 267 dated 19/06/03 "On the approval of laboratory practice rules".

The animals were distributed by the random sampling method into the following experimental groups: the intact group (n = 7), the control group with reproduction of the chronic pain model [8] (n = 7), the comparison group (B16/F10) – mice with standard subcutaneous transplantation of melanoma B16/F10 (n = 7), and the main group (chronic pain + B16/F10) – the B16/F10 melanoma was grafted 3 weeks after cre-

ating the chronic pain model (n = 7). In the mice of the main group (chronic pain + B16/F10), bilateral ligation of sciatic nerves was performed under the xylo-zoletil anesthesia. 3 weeks after healing of the surgical wound, 0.5 ml of suspension of B16/F10 melanoma tumor cells in the physiological solution diluted at 1:10 was introduced subcutaneously under the right shoulder blade. B16/F10 melanoma was grafted in the animals of the comparison group (B16/F10) subcutaneously at the same dosage and volume as that in the main group, but without reproducing the chronic pain model. In standard transplantation, the tumor emerges in 100% of cases, grows quite rapidly and metastasizes on days 12–16 into the lungs (60–90%) mainly hematogenously, less frequently it spreads into the liver and spleen. All manipulations with animals were performed in a sterile cabinet. The tools, utensils, and hands were disinfected by the conventional method.

All animals were decapitated by guillotine on day 14 of the experiment. After decapitation, the animals' skin was quickly excised with application of cooling agents, and mitochondria were isolated using the method of M.V. Egorov and S.A. Afanasyev [9]. In the obtained mitochondrial samples, the following levels were identified using the standard ELISA test systems: reduced glutathione (GSH) in nM/g protein (Bio Source, USA), oxidized glutathione (GSSG) in nM/g protein (Bio Source, USA), glutathione peroxidase-1 (GPx 1) in ng/mg protein (Ab Frontier, South Korea), glutathione peroxidase-4 (GPx 4) in ng/mg protein (Cloud-Clone Corporation, DPRK), glutathione reductase (GR) in ng/mg protein (Cusabio, DPRK), glutathione S-transferase (G-S-T) in ng/mg protein (Immundiagnostik, FRG), superoxide dismutase-2 (SOD-2) in pg/mg protein (Ab Frontier, South Korea), and total protein by the biuret method, g/l (Olveks Diagnosticum, Russia).

The statistical analysis of the results was performed using the Statistica 6.0 software package. The quantitative data for four groups (independent samples) were compared using the Kruskal – Wallis test with multiple comparison. The data are presented in the form of $M \pm m$, where M is the arithmetic mean value, and m is the standard error of the mean.

RESULTS

During the experiment, data were obtained on the influence of B16/ F10 melanoma, chronic neurogenic pain, and the combined effect of these pathological processes on the glutathione system in mitochondria of skin cells in female mice on the 14th day of the experiment – the logarithmic phase of the experimental tumor growth (Table 1). Higher concentrations of antioxidative system components were registered in mitochondria of skin cells in the animals with chronic neurogenic pain (the control group), as compared to the intact animals: GSH increased by 1.3 times, GPx 1 – by 2.9 times, GPx 4 – by 1.9 times, GR – by 2.8 times, and SOD-2 – by 2.4 times.

At the same time, malignant growth in animals of the comparison group led to completely opposite changes in the antioxidative system in the mitochondria: the content of GPx 1 decreased by 1.9 times, GPx 4 – by 3.7 times, GR – by 3.9 times, SOD-2 – by 3.8 times,

Table 1

Levels of antioxidant enzymes in mitochondria of skin cells in female mice during the growth of B16/F10 melanoma with chronic neurogenic pain

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
Reduced glutathione (GSH) (nM/g protein)	151 071.2 ± 717.41	196 860.1 ± 1 917.581 p^1= 0.000000	179 886.5 ± 15 147.22	219 446.3[1] ± 3 643.97 p^1 = 0.00000
Oxidized glutathione (GSSG) (nM/g protein)	462.32 ± 15.88	506.81 ± 19.83	637.25 ± 17.62[1,2] p^1= 0.000008 p^2 = 0.000356	714.34 ± 9.16[1,2] p^1 = 0.00000 p^2 = 0.000001
GPx 1 (ng/mg protein)	0.207 ± 0.009	0.604 ± 0.007[1] p^1 = 0.00000	0.107 ± 0.008[1,2] p^1 = 0.00005 p^2 = 0.00000	0.746 ± 0.014[1,2,3] p^1 = 0.00000 p^2 = 0.00001 p^3 = 0.00000
GPx 4 (ng/mg protein)	16.518 ± 0.216	32.263 ± 0.471[1] p^1 = 0.00000	4.435 ± 0.166[1,2] p^1 = 0.00000 p^2 = 0.00000	25.903 ± 0.282[1,2,3] p^1 = 0.00000 p^2 = 0.00000 p^3 = 0.00000

Table 1 (continued)

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
GR (ng/mg protein)	13.239 ± 0.190	36.717 ± 0.228[1] $p^1 = 0.00000$	3.403 ± 0.222[1,2] $p^1 = 0.00000$ $p^2 = 0.00000$	16.675 ± 0.189[2,3] $p^2 = 0.00000$ $p^3 = 0.00000$
Glutathione S-transferase (G-S-T) (ng/mg protein)	2.164 ± 0.127	1.898 ± 0.100	1.771 ± 0.099	2.433 ± 0.145[2,3] $p^2 = 0.010500$ $p^3 = 0.002727$
SOD-2 (pg/mg protein)	461.402 ± 20.133	1 128.2 ± 54.186[1] $p^1 = 0.00000$	120.84 ± 10.904[1,2] $p^1 = 0.00000$ $p^2 = 0.00000$	849.68 ± 32.492[1,2,3] $p^1 = 0.00000$ $p^2 = 0.00085$ $p^3 = 0.00000$

Note. 1 – statistically significant value compared to the values in intact animals; 2 – statistically significant value compared to the values in controls (chronic pain); 3 – statistically significant value compared to the values in the comparison group (B16/F10 tumor).

and GSSG rose by 1.36 times compared to the intact values. The same trend remained during comparison with the control animal group (chronic neurogenic pain). Here, the level of GPx 1 dropped by 5.6 times, GR – by 10.8 times, GPx 4 – by 7.3 times, and SOD-2 – by 9.3 times. The content of GSSG exceeded the control group values by 1.26 times. Meanwhile, GSH levels did not differ significantly from the intact and control values.

The combined effect of chronic neurogenic pain and tumor process contributed to an increase in production of GSH by 1.5 times, GPx 1 – by 3.6 times, G-S-T – by 1.28 times, GPx 4 – by 1.6 times, and SOD-2 – by 1.8 times, as compared to the values for the intact animal group. The content of GSSG was 1.54 times higher than the intact figures. As compared to the values in the animals suffering from chronic neurogenic pain only, the combined effect of two pathological processes exhibited 1.24 times higher level of GPx 1 ($p = 0.00001$), 2.2 times higher level of GR, 1.37 times higher level of G-S-T (at the level of the statistical trend), while SOD-2 and GPx 4 were 1.33 times and 1.24 times lower, respectively ($p = 0.00000$). The level of GSSG was 1.42 times higher, too. The comparison of a combination of chronic pain and tumor growth with tumor growth only (the comparison group) demonstrated a rise in GPx 1 by 6.9 times, GR – by 4.9 times, G-S-T – by 1.37 times, GPx 4 – by 5.8 times, and SOD-2 – by 7 times, respectively.

Table 2 presents ratios of the glutathione system components reflecting the maintenance of redox homeostasis in the animal organism.

The dominating inhibition of antioxidative components in tumor development and their excess in chronic pain introduced differently directed shifts into the function of physiological cascades of antioxidant enzymes. In particular, control animals (chronic neurogenic pain) demonstrated 1.22 times increase in the GSH/GSSG ratio and 2.2 times decrease in the GSH/GPx 1 ratio, as compared to the intact values. Tumor growth in the comparison group contributed to the reduction of the calculated ratios as follows: SOD-2/GPx 1 was 2 times lower, GR/GPx 1 – 2 times lower,

Table 2

Parameters of the glutathione cascade in mitochondria of skin cells in female mice during the growth of B16/F10 melanoma with chronic neurogenic pain

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
GSH/GSSG	3.2 ± 0.124	3.9 ± 0.130[1] $p^1 = 0.004849$	2.8 ± 0.300[2] $p^2 = 0.007518$	3.1 ± 0.053[2] $p^2 = 0.000064$
GSH/GPx 1	7.3 ± 0.298	3.3 ± 0.052[1] $p^1 = 0.000000$	17.8 ± 2.567[1,2] $p^1 = 0.001583$ $p^2 = 0.000102$	2.94 ± 0.039[1,2,3] $p^1 = 0.000000$ $p^3 = 0.000084$

Table 2 (continued)

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
SOD-2/GPx 1	2.2 ± 0.129	1.8 ± 0.100	$1.1 \pm 0.112^{1,2}$ $p^1 = 0.000036$ $p^2 = 0.000464$	$1.1 \pm 0.052^{1,2}$ $p^1 = 0.000004$ $p^2 = 0.000031$
GR/GPx 1	6.4 ± 0.258	6.0 ± 0.115	$3.2 \pm 0.324^{1,2}$ $p^1 = 0.000006$ $p^2 = 0.000003$	$2.2 \pm 0.063^{1,2,3}$ $p^1 = 0.000000$ $p^2 = 0.000000$ $p^3 = 0.008103$

Note. 1 – statistically significant value compared to the values in intact animals; 2 – statistically significant value compared to the values in controls (chronic pain); 3 – statistically significant value compared to the values in the comparison group (B16/F10 tumor).

while the GSH/GPx 1 ratio, by contrast, significantly increased by 2.44 times. As compared to the group with chronic neurogenic pain, the ratios in the group with tumor growth were reduced: the GSH/GSSG ratio fell by 1.4 times (p = 0.007518), SOD-2/GPx 1 – by 1.64 times, GR/GPx 1– by 1.87 times, while the GSH/GPx 1 ratio rose by 5.4 times. The combined effect of two pathological processes inhibited the function of physiological cascades of antioxidant enzymes. So, GSH/GPx 1 decreased by 2.5 times as compared to the intact values, SOD-2/GPx 1 – by 2 times and GR/GPx 1 – by 2.9 times, respectively. In comparison with the control group of animals (chronic neurogenic pain), changes were observed for the GSH/GSSG, SOD-2/GPx 1, and GR/GPx 1 ratios, which manifested themselves through the reduction of their values by 1.26 times (p = 0.000064), 1.64 times, and 2.7 times, respectively. The decrease in GSH/GPx 1 by 6 times and GR/GPx 1 – by 1.45 times was found, compared to the group of animals with tumor growth (the comparison group). Changes in the cascade reactions were associated with GPx 1 responsible for mechanical detoxification of peroxides performed with the help of the enzymatic bi-bi mechanism with two molecules of GSH. Due to this, enzymatic detoxification of non-radical hydroperoxides took place, and the oxidative-reductive balance was regulated directly by elimination of hydroperoxides and oxidation of GSH, the main low-molecular thiol in the cells [10]. The overabundance of GPx 1 is quite likely to result in a lack of potential substrates, which manifests itself through inhibition of all cascades with its participation.

DISCUSSION

Analyzing the obtained data, the authors believe that mitochondria of skin cells in the presence of chronic neurogenic pain respond towards the reductive stress, which manifests itself in the active production of all the enzymes studied, including the tripeptide – GSH. In the case of the neoplastic process in the skin mitochondria – the target tissue for melanoma – the classical scenario of the peroxide theory of carcinogenesis is observed [11] with inhibition of all antioxidant enzymes and accumulation of oxidized glutathione. Chronic neurogenic pain in animals with the tumor process prevents melanoma from changing the reductive stress that already developed. Due to this, all antioxidant enzymes are at quite high levels, but the enzymatic cascade interaction is inhibited, which is demonstrated by the values of the calculated ratios: GSH/GPx 1, GR/GPx 1. The excessive accumulation of reductive equivalents leads to reductive stress; it is also characterized by the absence of oxidants and/or by the decrease in excessive equivalents [12, 13]. The concept of reductive stress is rather new. For a certain time, the absence of cellular oxidants has been known to reduce the growth responses of cells. Newer pieces of evidence point to additional cellular and physiological effects caused by the absence of cellular oxidants and accumulation of excessive reductive equivalents, including changes in the formation of disulphide protein bond, reduced mitochondrial function, and lower cellular metabolism [13]. At present, a number of studies confirm that the “deoxidizing”, or reductive, stress accompanies such conditions as hypoxia and hyperglycemia, that inhibit the mitochondrial function and cause the excessive accumulation of cellular reducing equivalents [14–16]. The authors believe that chronic neurogenic pain belongs to the same conditions which can change the function of mitochondria (in this case, mitochondria of skin cells) towards reductive stress, and the trend remains if the neoplastic process joins. The increasing amount of antioxidant

enzymes under the effect of chronic neurogenic pain at the moment of tumor appearance may be at such a stable condition which cannot be reverted or inhibited by the oxidative process which, in its turn, characterizes the tumor growth.

CONCLUSION

The obtained results showed that chronic neurogenic pain has a modulating effect on the functioning of skin cell mitochondria, promoting a shift of their antioxidant system towards the reductive stress, which manifests itself through an essential increase in the content of antioxidant enzymes. Such a response of mitochondria of skin cells can result in other patterns in the development of the neoplastic process during chronic neurogenic pain. Definitely, it is only skin that is in question, and not the entire organism on the whole, and the authors realize that a functional response of mitochondria in different organs can vary. This is what causes interest and requires further research.

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Authors contribution

Neskubina I.V. – conception and design; analysis and interpretation of data. Frantsiyants E. M. – conception and design; final approval of the manuscript for publication. Surikova E.I. – analysis and interpretation of data. Kaplieva I.V. – conception and design. Trepitaki L.K. – conception and design. Nemashkalova L.A. – substantiation of the manuscript, critical revision for important intellectual content. Lesovaya N.S. – substantiation of the manuscript, critical revision for important intellectual content.

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Effects of smoking on the level of SP-A and SP-D surfactant proteins in the blood of patients without bronchopulmonary diseases

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ABSTRACT

Every year, about six million people die from tobacco use. Respiratory epithelium is the first line of defense against exogenous invasion, in particular, harmful inhaled particles, pathogens and allergens. However, the epithelium of the respiratory tract is also a regulator of immunological and inflammatory reactions through secretion of inflammation and immune cell recruitment mediators. An important component of the pulmonary immune system is the surfactant, and, in particular, its proteins SP-A and SP-D, synthesized mainly by type II pneumocytes.

Aim. To assess the levels of surfactant proteins SP-A and SP-D in the blood of smoking patients without bronchopulmonary diseases.

Materials and methods. The study included 59 patients admitted to the department of internal medicine with hypertension. The general group was divided into subgroups: non-smoking patients (n = 31) and healthy smokers (n = 28). All patients underwent clinical, functional, diagnostic and laboratory tests. The content of surfactant proteins SP-A and SP-D in the blood was determined by enzyme immunoassay.

Results. The subgroups did not differ in sex, age, height, body weight, blood pressure, heart rate, respiratory rate, and the distribution of comorbidities. The subgroups differed in the platelet level; in other main parameters of complete blood count and blood biochemistry no differences were revealed. It was found that the blood levels of surfactant proteins SP-A and SP-D in the subgroup of healthy smokers were significantly higher in comparison with the subgroup of non-smoking patients. The correlation analysis revealed a direct relationship between surfactant proteins SP-A and SP-D and smoking (R = 0.360, p = 0.006, R = 0.274, p = 0.037), a negative correlation between SP-D protein and age (R = –0.315, p = 0.016), and a direct relationship between SP-A protein and diastolic blood pressure (R = 0.271, p = 0.039). In the non-smoking subgroup, a negative correlation between SP-D and age (R = –0.438, p = 0.016) and between SP-D and systolic blood pressure (R = –0.433, p = 0.017) was identified.

Conclusions. The direct relationship between higher levels of the surfactant proteins SP-A and SP-D and smoking in the group of healthy smokers is justified (inflammatory changes, structural abnormalities in the lung parenchyma under the influence of cigarette smoke). The SP-D protein is more significant in comparison with the SP-A protein in vascular wall remodeling, lung tissue matrix, oxidative lung tissue damage, and apoptosis, which explains its negative correlation with age and systolic blood pressure.

Key words: surfactant, surfactant protein A, surfactant protein D, biomarker, smoking.

Conflict of interest. The authors declare the absence of obvious and 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 of the Research Institute of Therapy and Preventive Medicine (Protocol No. 15 of 10.04.2018).

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Влияние курения на уровни сурфактантных белков SP-A и SP-D в крови у пациентов без бронхолегочных заболеваний

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РЕЗЮМЕ

Актуальность. Ежегодно около 6 млн человек умирают из-за употребления табака. Дыхательный эпителий – первая линия защиты против экзогенной инвазии, в частности вредных вдыхаемых частиц, патогенов и аллергенов. Однако эпителий дыхательных путей является не просто физическим барьером, но и регулятором иммунологических и воспалительных реакций посредством секреции медиаторов воспаления и рекрутинга иммунных клеток. Важным компонентом легочной иммунной системы является сурфактант, в частности его белки SP-A и SP-D, синтезируемые в основном пневмоцитами II типа.

Цель. Оценить уровень сурфактантных белков SP-A и SP-D в крови у курящих пациентов без наличия бронхолегочных заболеваний.

Материалы и методы. В исследование включены 59 пациентов, госпитализированных в терапевтическое отделение по поводу гипертонической болезни. Общая группа разделена на подгруппы: некурящие пациенты (n = 31) и «здоровые курильщики» (n = 28). Всем пациентам проведены клиническое, функционально-диагностическое и лабораторное исследования. Содержание сурфактантных белков SP- A и SP-D в крови определяли методом иммуноферментного анализа.

Результаты. Подгруппы не различались по полу, возрасту, росту, массе тела, уровню артериального давления, частоте сердечных сокращений, частоте дыхательных движений, а также по распределению сопутствующей патологии. Сравниваемые подгруппы достоверно отличались по уровню тромбоцитов, по остальным основным параметрам общего анализа крови, биохимического анализа различий не отмечено. Выявлено, что уровень в крови сурфактантных белков SP-A и SP-D в подгруппе «здоровых курильщиков» достоверно выше в сравнении с подгруппой некурящих пациентов. При корреляционном анализе прямая связь получена для сурфактантных белков SP-A и SP-D и курения (R = 0,360; p = 0,006; R = 0,274; p = 0,037), Обратная корреляционная связь выявлена SP-D с возрастом (R= –0,315; p = 0,016) и прямая связь белка SP-A с диастолическим артериальным давлением ((R = 0,271; p = 0,039). В подгруппе некурящих получена обратная связь SP-D с возрастом (R = –0,438; p = 0,016) и систолическим артериальным давлением (R = –0,433; p = 0,017).

Заключение. Отмечены более высокий уровень сурфактантных белков SP-A и SP-D в группе курящих пациентов, их прямая связь патогенетически обоснована (воспалительные изменения, структурные аномалии в паренхиме легких при воздействии сигаретного дыма). Белок SP-D более значим в сравнении с SP-A при ремоделировании сосудистой стенки, матрикса ткани легкого, при окислительном повреждении ткани легкого и апоптозе, что объясняет его обратную связь с возрастом и систолическим артериальным давлением.

Ключевые слова: сурфактант, сурфактантный белок A, сурфактантный белок D, биомаркер, курение.

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

Источники финансирования. Материал статьи является частью бюджетной темы НИИТПМ – филиал ИЦиГ СО РАН «Эпидемиологический мониторинг состояния здоровья населения и изучение молекулярно-генетических и молекулярно-биологических механизмов развития распространенных терапевтических заболеваний в Сибири для совершенствования подходов к их диагностике, профилактике и лечению». Работа выполнена в рамках государственного задания по интеграционному проекту (0324-2018-0040) «Разработка новых способов экспресс-диагностики заболеваний человека на основе детекции органоспецифических маркеров с помощью современных физических и физико-химических подходов».

Соответствие принципам этики. Все пациенты подписали информированное согласие. Исследование одобрено локальным этическим комитетом НИИТПМ – филиал ИЦИГ СО РАН (протокол № 15 от 10.04.2018).

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INTRODUCTION

Despite the efforts aimed at decreasing prevalence of smoking, approximately six million people die due to tobacco consumption worldwide annually [1]. Cigarette smoking contributes greatly to the pathogenesis of chronic obstructive pulmonary disease (COPD), hypertension, cardiovascular and oncological diseases with inflammatory components, such as atherosclerosis, Crohn's disease, rheumatoid arthritis, psoriasis, Grave's ophthalmopathy, and non-insulin-dependent diabetes mellitus [2–5]. Apart from this, smokers show elevated sensitivity to microbial infections (respiratory tract infections, bacterial meningitis and periodontitis) and wound healing disorder [6]. Respiratory epithelium is the first line of defense against exogenous invasion including inhaled noxious particles, pathogens and allergens. However, respiratory epithelium is not merely a physical barrier, but also a regulatory mechanism for immune and inflammatory responses through secretion of inflammation and immune cell recruitment mediators [7, 8, 9]. An important component of the immune system is the surfactant and, in particular, its proteins SP-A and SP-D, mainly synthesized by type II pneumocytes [10].

Physiologically, small amounts of surfactant proteins SP-A and SP-D are found in blood. Tobacco smoke promotes increased alveolar-capillary leakage of surface-active proteins into the blood, and their level may facilitate assessment of damage to the lungs caused by smoke. The potential to use surfactant proteins as markers of alveolar epithelium damage against the background of smoking has not been studied previously and only rare investigations regarding SP-A and SP-D levels in patients with COPD have been conducted. Therefore, studying these mechanisms is relevant in modern medical science for identification of smoking individuals exposed to the risk of COPD.

The aim of the study was to assess the levels of surfactant proteins SP-A and SP-D in the blood of smoking patients without bronchopulmonary diseases.

MATERIALS AND METHODS

A total of 59 patients admitted to the department of internal medicine with hypertensive disease were enrolled in the study. The inclusion criteria were: worsening of hypertensive disease progression (the mean index of systolic arterial pressure (SAP) ≥140 mmHg during automatic evaluation of arterial blood pressure at the doctor's office), patients of both genders aged 18 to 75 years, absence of acute and chronic bronchial and pulmonary diseases, absence of changes in spirometry and x-ray scans of thoracic organs, and consent to participate in the study and fill in a respective informed consent form. The exclusion criteria were: presence of acute infectious processes at the moment of enrollment; presence of oncological diseases; previous chemotherapy or radial therapy; immunodeficiency disorders; previous/active pulmonary tuberculosis; clinically significant (according to judgment of the researcher) unstable cardiologic disease, e.g. uncontrolled symptomatic arrhythmia, atrial fibrillation, cardiac insufficiency with congestion phenomena of 3rd or 4th grades according to the NYHA classification; severe renal insufficiency diagnosed through evaluation of eGFR calculated using the CKD-EPI formula (Chronic Kidney Disease Epidemiology Collaboration) with consideration for creatinine concentration in the serum below 15 ml/min/1.73 m^2; type 1 diabetes mellitus (DM); pregnancy or lactation; and presence of a known life-threatening comorbidity with life expectancy < 18 months from the moment of enrollment into the study. The general group was divided into two subgroups: non-smoking patients (n = 31) and healthy smokers (n = 28). "Healthy smokers" is a

term used in modern literature meaning absence of respiratory symptoms or minimal respiratory symptoms (cough, expectoration, shortness of breath after insignificant physical loads) that may only be revealed using a clinical survey [11]. The subgroup of healthy smokers only included patients with the minimal smoking index of 2 packs/year.

All patients underwent clinical, functional, diagnostic, and laboratory assessment. Laboratory diagnosis (complete haemogram, blood biochemistry) was carried out using the biochemical analyzer Beckman Coulter AU 480 (Beckman Coulter, USA) and the haematology analyzer Siemens advia2120i, BC 5300 (Germany). The levels of the surfactant proteins SP-A and SP-D in the blood serum were evaluated by the method of immune-enzyme analysis using the Multiscan EX analyser (Finland) and the ELISABioVendor test system (R&D, USA). X-ray examination of thoracic organs was conducted using the TeleKoRD-MT device (a remotely operated diagnostic X-ray complex, Russia). The external respiration function was evaluated using the Spirolab I spirometer (Italy).

Statistical processing of the data obtained was performed using the SPSS 10.05 program package. The pattern of quantitative attribute distribution was determined using the Kolmogorov–Smirnov method. In case of normal distribution, the mean value (*M*) and standard deviation (*SD*) were calculated. The Student's *t*-test was used to compare normally distributed samples. In case of non-Gaussian distribution, the median (*Me*), and the 25 and 75 percentiles were calculated. Interrelations between the attributes were evaluated through calculation of the Spearman's correlation coefficient (*R*). The $\chi 2$ criterion was used for qualitative attributes. The critical level of statistical significance in the null hypothesis tests was assumed to be 0.05. The study protocol was approved by the local Ethics Committee at the research site.

RESULTS

The clinical characteristics of the patients are presented in Table 1.

The subgroups did not differ in sex, age, height, body mass, arterial blood pressure level, heart rate, respiratory rate or distribution of comorbidities.

The characteristics of patients' laboratory data (complete blood count, blood biochemistry, SP-A and SP-D surfactant protein levels) are presented in tables 2 and 3.

The compared subgroups were significantly different in thrombocyte levels; no difference was revealed in the remaining parameters of complete blood count and biochemistry.

Table 1

Clinical characteristics of the patients

Parameter	General group of patients, *n* = 59	Subgroup of healthy smokers, *n* = 28	Subgroup of non-smoking patients, *n* = 31	*p*
Sex, men/women, *n* (%)	32 (54.2) / 27 (45.8)	17 (60.7) 11 (39.3)	15 (48.4) / 16 (51.6)	0.421
Age, years, *Me* (25%; 75%)	55 (47; 68)	53 (48; 65)	61 (44; 68)	0.543
Height (*M* ± *SD*), cm	169.2 ± 9.0	170.6 ± 9.6	168.0 ± 8.3	0.113
Body weight, *Me* (25%; 75%), kg	79 (69; 85)	80 (71; 83)	75 (65; 86)	0.101
Systolic blood pressure (*M* ± *SD*), mmHg	157 ± 26	162.7 ± 26.8	152.3 ± 24.3	0.343
Diastolic blood pressure (*M* ± *SD*), mmHg	89 ± 12	91.8 ± 12.7	86.3 ± 10.5	0.320
Respiratory rate (*M* ± *SD*), beats per minute	17.6 ± 4.8	17.3 ± 6.4	18.0 ± 2.6	0.716
Heart rate (*M* ± *SD*), beats per minute	84.2 ± 10.2	83.4 ± 10.1	83.8 ± 10.4	0.113
Number of patients with type 2 diabetes, *n* (%)	8 (13.6)	4 (14.3)	4 (12.9)	0.885
Number of patients with obesity, BMI ≥ 30, *n* (%)	21 (35.6)	10 (35.7)	11 (35.4)	0.933

Note. *p* – significance of differences between the subgroups of non-smoking patients and healthy smokers. BMI – body mass index.

It was revealed that the blood level of surfactant proteins SP-A and SP-D in the subgroup of healthy smokers was significantly higher than in the subgroup of non-smokers.

Significant correlations in the general group of patients are presented in table 4.

A direct correlation was found between SP-A and SP-D surfactant proteins and smoking. An inverse correlation was revealed between the SP-D protein and age. Additionally, a positive correlation was found between the SP-A protein and systolic arterial blood pressure. While investigating the correlations in the subgroups separately, a negative correlation was found between SP-D and age (Spearman (*R*) -0.438, $p = 0.016$) and SP-D and diastolic blood pressure (Spearman (*R*) -0.433, $p = 0.017$) in the subgroup of non-smokers.

Table 2

Patients' laboratory data

Parameter	General group of patients, *n* = 59	Subgroup of healthy smokers, *n* = 28	Subgroup of non-smoking patients, *n* = 31	*p*
White blood cells, *Me* (25%; 75%), *10⁹/l	8.1 (6.6; 10.1)	9.0 (6.5; 10.5)	7.6 (6.6; 9.7)	0.427
Red blood cells (*M* ± *SD*), *10⁹/l	4.5 ± 0.7	4.5 ± 0.9	4.5 ± 0.5	0.737
Hemoglobin (*M* ± *SD*), g/l	135.3 ± 22.7	135.8 ± 27.7	134.8 ± 17.4	0.762
Platelets, *Me* (25%; 75%), *10⁹/l	225 (176; 267)	184 (150; 236)	249 (202; 268)	0.016
Erythrocyte sedimentation rate, *Me* (25%; 75%), mm/hour	8 (5; 13)	8 (4; 13)	9 (6; 12)	0.861
Alanine aminotransferase, *Me* (25%; 75%), ME/l	18.5 (12.0; 29.2)	21.5 (14.1; 33.7)	15.0 (11.3; 23.2)	0.069
Aspartate aminotransferase, IU (25%; 75%), IU/l.	21.3 (17.1; 35.0)	22.0 (18.3; 46.2)	20.5 (17.0; 30.1)	0.349
Total protein (*M*±*SD*), g/l	71.0 ± 26.5	72.6 ± 7.3	69.9 ± 5.7	0.146
Total bilirubin, *Me* (25%; 75%), mmol/l	13.7 (10.8; 17.4)	13.4 (9.8; 17.3)	14.2 (11.5; 17.4)	0.611
Cholesterol (*M* ± *SD*), mmol/l	4.8 ± 1.3	4.7 ± 1.1	4.8 ± 1.4	0.902
Creatinine, *Me* (25%; 75%), mmol/l	99.0 (80.9; 21.6)	100.0 (81.2; 133.4)	98.0 (78.0; 108.0)	0.237
Glucose, *Me* (25%; 75%), mmol/l	5.2 (4.6; 5.9)	5.3 (4.9; 6.0)	5.0 (4.4; 5.8)	0.221
Urea, *Me* (25%; 75%), mmol/l	6.1 (5.4; 8.5)	5.9 (4.8; 8.9)	6.3 (5.5; 8.1)	0.809

Note. *p* – significance of differences between the subgroups of non-smoking patients and healthy smokers.

Table 3

SP-A and SP-D surfactant protein levels

Parameter	General group of patients, *n* = 59	Subgroup of healthy smokers, *n* = 28	Subgroup of non-smoking patients, *n* = 31	*p*
SP-A, *Me* (25%; 75%), (ng/ml)	34.19 (26.97; 45.96)	44.60 (28.35; 61.56)	29.26 (21.25; 39.46)	0.007
SP-D, *Me* (25%; 75%), (ng/ml)	274.06 (173.95; 484.22)	333.99 (232.32; 593.35)	242.37 (145.51; 356.80)	0.039

Note. *p* – significance of differences between the subgroups of non-smoking patients and healthy smokers.

Table 4

Significant correlations in the general group of patients

Correlation pair	General group of patients, *n* = 58	
	Spearman (*R*)	*p*
SP-A – Smoking	0.360	0.006
SP-D – Smoking	0.274	0.037
SP-A – Diastolic blood pressure	0.271	0.039
SP-D – Age	–0.315	0.016

DISCUSSION

The obtained results regarding higher blood levels of SP-A and SP-D surfactant proteins in the group of healthy smokers in comparison with the subgroup of non-smoking patients comply with the results obtained by Sorensen G.L. et al. (2006), Mazur W. et al. (2011), Behera D. et al. (2005), Helen Ilumets et al. (2011), Moazed F. et al. (2016), and Nida, Lone (2018) [12–17]. Non-smokers usually demonstrate inflammatory changes and structural abnormalities in respiratory ways and parenchyma caused by cigarette smoke and leading to passage of SP-A and SP-D surfactant proteins into blood [18]. This is associated with loss of blood–air barrier integrity against the background of smoking, which is responsible for the leak of the secreted pulmonary proteins into the blood channels through the vessels [19]. It has been demonstrated in experiments that the gradient of SP-A and SP-D concentration makes it possible for proteins synthesized in the respiratory tract to leak into the blood flow against the background of exposure to cigarette smoke [16, 20, 21]. In certain circumstances, including acute exposure to cigarette smoke, the level of surfactant proteins may decrease in the bronchoalveolar lavage fluid while simultaneously increasing in the blood serum. The smoking status is a strong predictor of such translocation [16, 22–24].

In our study, a strong association between SP-D and SABP in the subgroup of non-smoking patients is worth noting. In the literature available to us, there was no reference to the association between surfactant proteins SP-A and SP-D and systolic or diastolic arterial blood pressure. It is known that hypertensive angiopathy essentially involves vascular remodeling: a complex structural and spatial modification of small arteries, including lung tissues [25–27]. Wall remodeling is a multi-layer interaction including hypertrophy, hyperplasia, apoptosis, hyalinosis, and fibrinoid necrosis of smooth muscle cells as well as deposition of extracellular matrix [28, 29]. An important role of SP-A and SP-D proteins in apoptosis regulation, further digestion of cell debris by phagocytes and subsequent remodeling of extracellular matrix has been proved in experiments. However, SP-D is a more potent modulator of pulmonary cell apoptosis in comparison with SP-A [30]. Therefore, not only impairment of alveolar-capillary permeability in the lungs is observed against the background of higher arterial blood pressure, but also active participation of SP-D in vessel wall remodeling, which may affect downregulation of this protein in blood.

In our study, the inverse correlation of SP-D blood level and age was shown, while there was no correlation between obesity and SP-D or SP-A. Research in this field is rare and inconsistent. Thus, according to the study by Sorensen G.L et al. (2006), age and obesity were outlined as important determinants of constitutional SP-D circulation levels [12]. This is explained by the experimentally demonstrated association between the alveolar SP-D level elevation and increased oxidative damage to lung tissue [31]. Studies by Betsuyaku T. et al. (2014) and Zhao X.M. et al. (2007) devoted to the alveolar SP-D level in humans showed no significant change in it with age [32, 33]. These findings comply with the data by Moliva J.I. (2014) revealing that no alveolar SP-D induction was observed with increasing age alongside with cytokine and oxidant induction [34].

Thus, a positive correlation between higher indices of SP-A and SP-D surfactant proteins in the group of smokers was pathogenetically substantiated. The SP-D protein is more important than the SP-A protein for remodeling of the vessel wall and lung tissue matrix as well as oxidative damage to lung tissue and apoptosis, which explains its inverse correlation with age and systolic arterial blood pressure.

CONCLUSION

The levels of SP-A and SP-D surfactant protein in smoking patients without bronchopulmonary diseases were significantly higher in comparison with non-smoking patients. The SP-A protein level has inverse correlation with the age and systolic arterial blood pressure of the patient.

Further research is required in order to determine whether SP-A and SP-D could be used as markers for early identification of smokers exposed to the risk of COPD.

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Authors contribution

Kharlamova O.S. – collection and processing of the materials, statistical processing, analysis and interpretation of data, drafting of the manuscript. Nikolaev K.Yu., Voevoda M.I. – conception and design, analysis and interpretation of data, editing of the manuscript. Ragino Yu.I. – conception and design, collection of data for analysis, analysis and interpretation of data, editing.

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Inflammatory activity and markers of extracellular matrix destruction in pulmonary tuberculoma

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ABSTRACT

Aim. To correlate the concentration of markers of extracellular matrix (ECM) destruction in peripheral blood with morphological characteristics of inflammatory activity and to evaluate their applicability in determining treatment strategy for patients with pulmonary tuberculoma (TUB).

Materials and methods. Peripheral blood samples were taken from 87 patients diagnosed with TUB. The concentrations of matrix metalloproteinases (MMPs), such as collagenases (MMP-1 and MMP-8), stromelysin (MMP-3), gelatinase (MMP-9), and tissue inhibitors of metalloproteinases (TIMP-1), were measured using the ELISA method (R&D Systems, Minneapolis, MN, USA). The activity of α_2-macroglobulin (MG), neutrophil elastase (NE) and proteinase inhibitor (PI) were measured using enzyme assays; acute phase reactants (APR) – haptoglobin (GP) and α_1-acid glycoprotein (AGP) – were measured using immunoturbidimetric assays (Thermo Fisher Scientific, USA). Statistica 7 software package and the predictive classification method (PCM) were employed for data analysis.

Results. It has been established that TUB as a clinical form of pulmonary tuberculosis (TB) is characterised by enzyme imbalance between MMP, NE and their inhibitors, namely, by an increase in the levels of MMP-1, MMP-8, MMP-9, and NE and a decrease in MG without changes in MMP-3, TIMP-1 and PI. There is a clear correlation between markers of ECM destruction in blood and morphological characteristics of inflammatory activity. The combinations of MMP-1 and MG can serve as a diagnostic criterion for caseous necrosis in the TUB centre (the alterative component of inflammation), while the levels of MMP-8 and MG can be indicative of granulomatous changes in the capsule (the productive component of inflammation). Various combinations of markers of ECM destruction (with or without APR) enable to predict a particular morphological pattern with accuracy from 80% up to 92%.

Conclusions. When determining a treatment strategy for patients with TUB, biochemical data which allow to assess the tempo and intensity of the inflammation process should be taken into account along with a dataset of clinical and radiological features.

Key words: extracellular matrix, matrix metalloproteinases, proteinase inhibitors, pulmonary tuberculoma.

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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 St. Petersburg Scientific Research Institute of Phthisiopulmonology.

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Активность воспалительного процесса и маркеры деструкции внеклеточного матрикса при туберкулеме легких

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РЕЗЮМЕ

Цель. Сопоставить уровень маркеров деструкции внеклеточного матрикса (ВКМ) в периферической крови с морфологическими характеристиками активности воспалительного процесса и определить возможность их использования при выборе тактики лечения больных с туберкулемой легких (ТУБ).

Материалы и методы. В периферической крови 87 больных (55 мужчин и 32 женщины) с верифицированным диагнозом ТУБ иммуноферментным методом определяли концентрацию коллагеназ (матриксные металлопротеиназы (ММП) 1, 8), стромелизина (ММП-3), желатиназы (ММП-9), тканевого ингибитора ММП-1 (ТИМП-1) с использованием наборов R&D Systems (США); энзиматически – активность нейтрофильной эластазы (НЭ), протеиназного ингибитора (ПИ) и α2-макроглобулина (МГ); иммунотурбодиметрически – концентрацию реактанты острой фазы воспаления (РОФ): гаптоглобина (ГП), α1-кислого гликопротеина (АГП) с использованием наборов Termo Fisher Scientific (США). Применяли пакет программ Statistica 7 и метод проективной классификации.

Результаты. Установлено, что ТУБ как клиническая форма туберкулеза легких характеризуется нарушением баланса ММП и НЭ с ингибиторами: повышением уровня ММП-1, -8, -9, НЭ и снижением МГ при отсутствии изменений ММП-3, ТИМП-1 и ПИ. Показано соответствие маркеров деструкции ВКМ в крови морфологическим характеристикам активности процесса. Информативными показателями для оценки альтернативного компонента воспаления (наличия казеоза в центре ТУБ) и его продуктивного компонента (гранулематозных изменений в капсуле) является как сочетание ММП-1 с МГ, так и ММП-8 с МГ. Различные комбинации показателей маркеров деструкции ВКМ (в сочетании с РОФ или без) дают возможность прогнозировать ту или иную морфологическую картину с точностью 80–92%. **Заключение.** При выборе тактики лечения больных с ТУБ следует принимать во внимание биохимические данные с их оценкой активности воспалительного процесса наряду с комплексом клинико-рентгенологических характеристик.

Ключевые слова: внеклеточный матрикс, матриксные металлопротеиназы, ингибиторы протеиназ, туберкулема легких.

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. Обследования выполнены с информированного согласия пациентов. Исследование одобрено локальным этическим комитетом СПб НИИФ.

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INTRODUCTION

The search for various markers that would enable early diagnosis and prediction of treatment effectiveness for various pathologies has not lost relevance. The complexity of the problem is explained by the fact that most of the markers are highly sensitive, yet not specific enough. This determines the potential of their aggregate isolation and as a clinical prognostic tool [1]. Currently, one of the methods for assessing the intensity of the inflammatory and destructive process is evaluating the activity of various classes of blood proteinases: serine, cysteine, aspartic, and matrix metalloproteinases (MMPs). The latter are divided into several subgroups based on their substrate specificity: collagenases, gelatinases, stromelysins, etc. [2]. Proteins of the MMPs perform a double role in the pathogenesis of inflammation, causing the destruction of all components of the ECM and regulating the immune response in the inflammatory processes. The ultimate effect of the proteolytic systems depends on the proteinase and proteinase inhibitor correlation. Neutrophils, monocytes, macrophages, fibroblasts, and epithelial cells are the sources of MMPs. At the post-translational level, serine proteinases are involved in the pro-enzyme activation, while specific tissue inhibitors (TIMPs) and α_2-macroglobulin (MG) regulate their activity [3]. *Mycobacterium tuberculosis* (MTB) regulates matrix metalloproteinase gene expression along with tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1) [4].

A correlation between the morphological characteristics of the inflammatory activity and the functional and metabolic properties of phagocytes in different forms of pulmonary tuberculosis (PT) has been reported in a few works [5], while data on their association with the markers of ECM destruction cannot be found in the literature.

Pulmonary tuberculoma (TUB) is a clinical manifestation of secondary PT. The term TUB is used to describe a caseous necrotic mass located in the lungs, over 12 mm in diameter, encapsulated by multiple layers of fibrous connective tissue. In the capsule, single Langhans cells, surrounded by epithelioid cell tubercles (in the case of rapidly progressing inflammation process), are sometimes found. However, the progression of TUB may be slow and torpid [6]. According to the National Clinical Guidelines for Surgical Treatment of PT, surgery is recommended for TUB after 4–6 months of chemotherapy with no effect [7]. The absence of clinical and radiological signs of disease activity does not exclude the presence of its morphological manifestations. In recent years, the morphological features of TUB have become especially well known. It is mainly determined by the fact that the evidence of mass lesions serves as an indication for surgery, while the resected sections of lung tissue undergo a thorough pathological examination [8].

The aim of the study was to correlate the changes in the markers of ECM destruction (the activity of MMPs and serine proteinase) in peripheral blood with the morphological characteristics of the inflammation process and to evaluate their applicability in determining a treatment strategy for patients with TUB.

MATERIALS AND METHODS

The study included 87 patients (55 men and 32 women) diagnosed with TUB (based on clinical assessment and morphological examination), treated at St. Petersburg Scientific Research Institute of Phthisiopulmonology, Department of Thoracic Surgery. The average age of the patients was 35.3 ± 1.2 years. All the study participants were eligible for surgical treatment (2011–2017). The control group consisted of 20 healthy donors whose demographic characteristics were consistent with those in the patient cohort. In most cases, TUB formed due to the involution of infiltrative PT (95%) following chemotherapy for up to 1.5 years. CT scanning of the chest cavity revealed that TUBs located in the upper lobe, lower lobe, and bilaterally account for 70.2%, 17.2%, and 12.5% of cases, respec-

tively. TUBs with a size of 1–2 cm, 2–4 cm and more than 4 cm were found in 57.14%, 28.51% and 14.35% of cases, respectively. Before treatment, bacteriological examination of the sputum was performed, and MTB, mainly multidrug-resistant strains (MDR), was detected in 34.9% of cases, which is typical of present-day tuberculosis, regardless of its clinical and anatomical forms [9].

Biochemical tests were performed no earlier than 7 days prior to the surgery. The enzyme-linked immunosorbent assay (ELISA) was used to measure the MMP concentrations in blood serum with R&D Systems reagents (Minneapolis, MN, USA). Representatives of three MMP subfamilies – collagenases MMP-1 and MMP-8; gelatinase MMP-9; and stromelysin MMP-3 – were measured, as well as their inhibitor TIMP-1. The concentrations of acute-phase reactants (APR) – haptoglobin (GP) and α_1-acid glycoprotein (AGP) – were determined using immunoturbidimetric assays (Thermo Fisher Scientific, USA), according to the manufacturer's protocols. The enzyme methods were used to assess the activity of serine proteinase – neutrophilic elastase (NE) [10], proteinase inhibitor (PI), and MG [11].

All TUBs showed morphological features of caseoma (Table 1)*. The inflammatory activity was assessed according to the B.M. Ariel classification (1998) [6], based on the correlation between the parameters of caseous necrosis, capsules and surrounding lung tissue.

Table 1

Morphological characteristics of TUB inflammatory activity

Morphological characteristics		Characteristic frequency (absolute (*n*), relative %)
Number of tuberculomas	Single	35 (40.2)
	Multiple	41 (47.0)
	Conglomerate	11 (12.8)
Parameters of caseous necrosis	Without melting	23 (26.8)
	With melting	64 (73.2)
Capsule changes	One layer	34 (39)
	Two layers	53 (61)
Inflammatory activity level	2	23 (26)
	3	52 (45)
	4	24 (27.5)
	5	1 (1.5)

For statistical data analysis, the Statistica 7.0 software package was used. Qualitative attributes were presented in the form of absolute (*n*) and relative values (%). The indicators were presented as medians (*Me*) and the interquartile range (25%; 75%) ([Q_1; Q_3]). For a number of indicators, logarithmic data transformation – $\log_2 (x + 1)$ (*MeL*) – was used to reduce the skewness of distributions. The significance of the relationship between qualitative variables was tested using the Fisher's exact test. The hypothesis of homogeneity was estimated for two and several samples according to the criteria of the Mann–Whitney U test and the Kruskal – Wallis test, respectively. Correlation analysis was performed using the Spearman's rank correlation coefficient. Differences in indicators were considered significant at a level of statistical significance $p < 0.05$.

The objective assessment of morphological data was carried out through analysis of a set of ECM destruction markers using the predictive classification method (PCM) with a linear discriminant analysis algorithm. The advantage of the method is the possibility of analysis regardless of the completeness of the data presented [12]. Typically, the discriminant function (DF) is calculated simultaneously for all variables. PCM computes the set of most significant correlating DFs, constructed from different subsets of markers. Due to the small number of markers included in the DFs, they are easier to interpret. Moreover, this allows to examine the diversity of biochemical manifestations of the studied process from different points of view. Discriminant weights (standardised coefficients) enable identification of the variables that contribute the most to the discrimination between groups. Positive DF values in patients provide evidence to assign them to a group of patients with less severe manifestations of the disease.

RESULTS

TUB, as a clinical form of PT, was characterised by a moderate increase in blood concentrations of collagenases (MMP-1 and MMP-8) and a significant increase in gelatinase (MMP-9), while concentrations of stromelysin (MMP-3) and TIMP-1 remained at the control level. Additionally, there was a decrease in the activity of another MMP inhibitor – MG. A statistically significant increase in the activity of serine proteinase (NE) was established with no changes in its inhibitor (PI) activity (Table 2)*.

Table 2

Concentrations of the analysed biochemical parameters in patients with TUB, *Me* and *MeL* [Q_1; Q_3]

Marker	Patients with TUB *n* = 87	Healthy donors *n* = 20
MMP-1L (ng/mL)	1.74 [1.31; 2.30] *p* = 0.002	1.17 [0.89; 1.72]

Table 2 (continued)

Marker	Patients with TUB $n = 87$	Healthy donors $n = 20$
MMP-8L (ng/mL)	3.27 [2.64; 3.94] $p = 0.003$	2.58 [2.22; 2.70]
MMP-9 (ng/mL)	1638.00 [950.80; 2557.69] $p = 0.00004$	71.99 [51.33; 73.94]
MMP-3L (ng/mL)	1.55 [1.07; 2.16]	1.87 [1.57; 2.07]
TIMP-1L (ng/mL)	6.72 [6.58; 6.89]	6.66 [6.55; 6.80]
MG (ME)	1.70 [1.40; 2.16] $p = 0.00003$	3.00 [2.46; 3.28]
NE (ME)	195.60 [173.90; 217.30] $p = 0.0002$	163.00 [152.10; 173.90]
PI (ME)	1.82 [1.27; 2.17]	1.20 [0.91; 1.31]
GP (g/L)	1.56 [1.08; 2.14] $p = 0.01$	1.04 [0.90; 1.10]
AGP (g/L)	1.10 [0.86; 1.69]	0.96 [0.88; 1.08]

Note: TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; TIMP-1 – tissue inhibitor of metalloproteinases; MG – α_2-macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; GP – haptoglobin; AGP – orosomucoid; p – a statistical significance threshold compared to healthy donors (Mann – Whitney U test).

We found a direct correlation between the concentrations of MMP-9 and MMP-8 ($r = 0.44$, $p \leq 0.014$) and the NE activity ($r = 0.23$, $p \leq 0.05$), as well as between the levels of TIMP-1 and MMP-9 ($r = 0.31$, $p \leq 0.009$). Given the ability of NEs to act as a pro-MMP activator in the blood, it can be assumed that the absence of changes in the concentrations of its main inhibitor PI indirectly contributed to the growth of MMP-1. A negative correlation ($r = -0.46$, $p \leq 0.004$) between PI and MMP-1 [13] was found. According to the significant differences between the increase in MMP-1 and MMP-8 concentrations and a decrease in TIMP-1 in the blood, the proteolytic processes intensified as the TUB size grew. Consequently, the degradation of ECM during the formation of TUB was associated with an imbalance between proteinases and inhibitors, namely, the prevalence of the two classes of proteinase (MMP and NE) concentrations. This information is consistent with the reports stating that mycobacterial infection is able to enhance the expression and secretion of MMP, but not TIMP-1 [14].

The statistical analysis showed that morphologically, neither gender (Fisher's test), nor age or duration of chemotherapy (Kruskal – Wallis test) correlate with the intensity of the TUB inflammation process: all results had statistical significance over 0.70. At the same time, analysis of the interrelation between the biochemical parameters of destruction and the morphological signs of the inflammatory activity revealed associations with changes in the MMPs levels (Table 3)*.

Table 3

Concentrations of the analysed biochemical parameters with regard to the morphological sings of the inflammatory activity in TUB, Me and *MeL* [Q_1; Q_3]

Marker	Parameters of caseous necrosis (Signs of melting)		Changes in the capsule	
	No $n = 23$	Yes $n = 64$	Single-layered $n = 34$	Double-layered $n = 53$
MMP-1L (ng/mL)	1.59 [1.10; 1.83]	1.96 [1.48; 2.46] $p = 0.04$	1.82 [1.57; 2.40]	1.78 [1.26; 2.69]
MMP-8L (ng/mL)	3.28 [2.87; 3.87]	2.96 [2.46; 3.52]	2.84 [2.62; 3.26]	3.37 [2.92; 3.73] $p = 0.02$
MMP-9 (ng/mL)	1575.63 [916.30; 2195.86]	2,060.67 [913.68; 2,988.27]	1,647.07 [936.91; 2,228.03]	1,623.43 [916.29; 2,253.56]
MMP-3L (ng/mL)	1.56 [1.10; 1.84]	1.50 [0.86; 2.13]	1.41 [0.87; 1.77]	1.64 [1.07; 2.14]
TIMP-1L (ng/mL)	6.70 [6.60; 6.86]	6.66 [6.57; 6.86]	6.79 [6.65; 6.82]	6.64 [6.65; 6.93]
МГ (ME) MG (ME)	1.84 [1.40; 2.12]	2.20 [1.36; 3.09] $p = 0.04$	1.82 [1.6; 2.22]	2.11 [1.44; 2.72] $p = 0.04$
NE (ME)	197.33 [173.90; 217.30]	205.68 [162.90; 241.83]	196.61 [179.30; 217.30]	201.02 [168.43; 225.47]
PI (ME)	1.69 [1.27; 2.15]	1.80 [1.55; 2.09]	1.63 [1.45; 1.84]	1.66 [1.22; 2.15]

Note. TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; TIMP-1 – tissue inhibitor of metalloproteinases; MG – α_2-macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; GP – haptoglobin; AGP – orosomucoid; * p – a statistical significance threshold between the groups (Mann – Whitney U test).

With perceptible signs of melting, the values of MMP-1 and MG were higher (although the latter was lower than the control level in both cases) than with no signs of melting. The obtained results appear logically conclusive, as the centre of the granuloma, represented by caseous masses, is formed from destroyed macrophages that die upon contact with the MTB and release of proteinases, which violates the protease/antiprotease balance. This is consistent with the opinion of A. Kubler (2015) on the leading role of MMP-1 in the formation of the caseous centre [15].

A double-layered capsule composed of collagenous connective tissue (in the outer layer) and granulation tissue with macrophages, epithelioid cells and Langhans cells (in the inner layer) is formed during the transition from stabilisation to progression phase. Compared to the single-layered fibrous capsule, the double-layered one is associated with a more pronounced increase in MMP-8 / MG values. This is consistent with the published data on the increase in the number of granulocytes and their phagocytic activity with active TUBs [5].

Similarly, in contrast to the inflammation process of moderate intensity (level 3 of disease activity) and low intensity (level 2 of disease activity), acute inflammation process of high intensity (levels 4 and 5 of disease activity) revealed an increase in the concentrations of MMP-1 ($p = 0.03$) and MMP-9 ($p = 0.04$) and a decrease in the MG activity ($p = 0.003$). Besides, the values of MMP-3, TIMP-1 and PI were within the specific reference ranges, while the concentrations of NE were above the upper end of the reference range, regardless of the intensity of the inflammatory activity in terms of morphology and its constituent characteristics (Kruskal–Wallis test).

Consequently, the progression of the inflammation process is reflected in a more pronounced imbalance in the proteinase/inhibitor system, which is consistent with the literature data [16]. Moreover, this is true for all patients with at least one TUB lesion. In other words, the number of TUBs is irrelevant to the case.

PCM was used to classify the patients according to the degree of the inflammatory activity level. 8 markers of ECM destruction were analysed. For a more complete assessment, the analysis included data on the status of such multifunctional APRs as GP (one of its functions is the activation of pro-MMP-1) and AGP (it activates fibrogenesis) (Table 2). 32 DFs, separating patients with the lowest inflammatory activity level 2 (DF1) and the highest levels 4 and 5 (DF2) from the rest of the patients with the accuracy 80–92%, were obtained as outcome indicators of PCM associated with different levels of disease activity (Table 4)*.

Table 4

The most informative combinations of the analysed biochemical parameters, which determine the accuracy of inflammatory activity assessment in TUB

Parameters	Most informative combinations of concentrations of the analysed biochemical parameters and their weight	Level of inflammatory activity	Prediction accuracy (%)
DF 1	MMP-8 (1.13). NE (0.02). PI (–1.03).	2/3–5	86
	MMP-8 (1.34). MG (1.14). AGP (–1.29).	2/3–5	83
DF 2	MMP-1(0.55). MMP-3 (0.089). MG (–1.53)	2–3/4–5	92

Note. TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; MG – α_2-macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; AGP – orosomucoid.

Taking into consideration high interdependence among the DFs, determined by the method of their construction, we will limit ourselves to interpreting only a few of them. Separation of the patients with low-level inflammatory activity (level 2 of disease activity) from the rest of the patient population (levels 3 to 5) reflects neutrophil characteristics (DF1). This is characterised by lower values of neutrophil collagenase (MMP-8) and neutrophil degranulation marker (NE) in combination with a high concentration of its inhibitor (PI) according to the values of the correlation coefficient between proteinases and the inhibitor. When we consider the progression from inflammation of low and moderate intensity (levels 2-3 of disease activity) to severe inflammation (levels 4-5 of disease activity) (DF2), the combination of the concentrations of MMP-1 and stromelysin is of utmost significance. This is consistent with the published reports on the decisive role of the MMP-3 / TIMP-1 correlation in the destruction of ECM [17].

The use of PCM for assessing the morphological signs of the inflammation process enabled to identify the areas typical of patients with level 2 and levels 4–5 of the disease activity. Classification of patients with level 3 of the disease activity posed the greatest challenge. The disease in this group of patients may develop in either direction. This indicates not so much the limitations of the method as the unavoidable simplification of the mathematical model of the polymorphic picture of the inflammation process. Only two

morphological parameters – 'the nature of the caseous masses' and 'the state of the capsule' – were taken into account. Apparently, for a clearer classification, it is required to analyse more data with the inclusion of additional variables, such as screenings in the surrounding lung tissue, bronchial lesions, and regional lymph nodes.

With the example of TUB, the applicability of the PCM for assessing the inflammatory activity with the use of combinations of three ECM destruction markers (with or without APR) with prediction accuracy of 80-92% was shown. The proposed method enables a deeper understanding of a variety of biochemical manifestations of tissue and cellular inflammatory mechanisms. The absence of significant differences in most indicators of ECM destruction in their isolated assessment based on the morphological characteristics does not exclude their significant contribution to the formation of different morphological features.

In conclusion, it should be noted that when choosing the treatment strategy for patients with TUB, biochemical data assessing the intensity of the inflammation process should be taken into consideration along with a set of clinical and radiological features.

CONCLUSION

TUB, as a clinical form of secondary PT, is characterised by an increase in the levels of different classes of proteinases in peripheral blood. This contributes to a shift in the proteinase/inhibitor system balance towards proteinases. An increase in the concentrations of collagenases (MMP-1, MMP-8) and gelatinase (MMP-9) is observed, while stromelysin (MMP-3) and TIMP-1 remain at the control level along with low MG activity and uncompensated increase in the NE level.

There is a correlation between the markers of ECM destruction in peripheral blood and the morphological characteristics of the disease activity. Thus, the combination of MMP-1 and MG is an indicator for assessing the alternative component of inflammation (presence of caseous necrosis in the TUB centre), while the MMP-8 / MG system serves as a diagnostic criterion for its productive component (granulomatous changes in the capsule).

Using TUB as an example, the applicability of PCM for assessing the morphological features of the inflammation process using a number of combinations of three markers of ECM destruction (with or without APR) in peripheral blood with prediction accuracy of 80–92% was shown. They can be used as additional criteria in determining the treatment strategy for patients with TUB.

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Authors contribution

Esmedlyaeva D. S. – conception and design, preparation of samples, collection of materials, carrying out of biochemical research, analysis of literature, analysis and interpretation of data, drafting of the manuscript. Alekseeva N. P. – statistical analysis of the findings, drafting of the manuscript. Novitskaya T. A. – morphological research. Dyakova M. E. – biochemical research. Ariel B. M. – interpretation of data, drafting of the manuscript. Grigoriev I.V. – conception, editing of the manuscript. Sokolovich Ye. G. – conception, final approval of the manuscript for publication.

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The role of IL-33/ST2 system in the modulation of the immune response in infective endocarditis (a literature review)

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ABSTRACT

An inflammatory process accompanied by a considerable number of pathological conditions in the body is one of the symptoms of infective endocarditis. The components of the immune system involved in the inflammatory response may serve as markers determining the development and prognosis of the disease and as potential therapeutic targets. These components include cytokines IL-33, sST2, and the IL-33/ST2 system, which are actively involved in the modulation of the inflammatory response. At present, the role of these biologically active molecules is well described for various pathologies associated with tissue destruction, including cardiovascular diseases, but not for the pathogenesis of infective endocarditis. This review is aimed at analyzing the available information on the pathogenesis of infective endocarditis, the role of IL-33 and ST2 in the formation of the inflammatory response in various pathological processes, and changes in the expression of the genes encoding these proteins under the influence of various factors.

Key words: IL-33, ST2, interleukin, infective endocarditis, cardiovascular diseases, protein secretion, gene expression.

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Роль комплекса IL-33/ST2 в модуляции иммунного ответа при инфекционном эндокардите (обзор литературы)

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РЕЗЮМЕ

Процесс воспаления, который сопровождает немалое количество патологических состояний организма, является одним из формирующих комплекс симптомов инфекционного эндокардита факторов. Компоненты иммунной системы, участвующие в воспалительном ответе, могут являться маркерами, определяющими развитие и прогноз заболевания, а также могут быть потенциальными терапевтическими мишенями. К таким компонентам относятся цитокины IL-33, sST2 и рецепторный комплекс IL-33/ST2, принимающие активное участие в модулировании воспалительной реакции. На настоящий момент роль этих биологически активных молекул достаточно хорошо описана для различных патологий, связанных с деструкцией тканей, в том числе и при сердечно-сосудистых заболеваниях, но не для патогенеза инфекционного эндокардита.

Данный обзор направлен на анализ имеющейся информации о патогенезе инфекционного эндокардита, роли IL-33 и ST2 в формировании воспалительного ответа при различных патологических процессах и экспрессии генов, кодирующих эти белки под воздействием различных факторов.

Ключевые слова: IL-33, ST2, интерлейкин, инфекционный эндокардит, сердечно-сосудистые заболевания, секреция белка, экспрессия гена.

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

Источник финансирования. Работа выполнена при поддержке комплексной программы фундаментальных научных исследований СО РАН в рамках фундаментальной темы НИИ КПССЗ № 0546-2015-0011 «Патогенетическое обоснование разработки имплантатов для сердечно-сосудистой хирургии на основе биосовместимых материалов, с реализацией пациент-ориентированного подхода с использованием математического моделирования, тканевой инженерии и геномных предикторов».

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INTRODUCTION

The morbidity of infective endocarditis (IE) even in economically prosperous countries of Western Europe and the USA ranges from 25 to 93 per 1 million people, and the mortality rate from this disease remains high – from 18 to 36% according to various sources [1]. The incidence of IE in the Russian Federation is 3–10 cases per 100 thousand people per year [2]. Undoubtedly, IE is a heterogeneous disease characterized by a wide range of clinical manifestations, which depend on both an etiological agent and a complex of predisposing factors.

Based on the notion of IE as a systemic disease, the severity of which is largely determined by the immunopathological processes associated with invasion and elimination of the pathogen, the primary task in determining the ways of its prevention lies in the search for key immunological factors that determine the body's resistance to pathogenic and opportunistic microorganisms. Components of the immune system, such as cytokines, immunoglobulins, components of the complement system, and others, are active participants in the inflammatory response induced by the introduction of a microbial agent. A key way to initiate an inflammatory response is to activate NF-kB and MAPK signaling pathways, pathogen-associated molecular patterns (PAMPs) of microorganisms, and some cytokines (e.g., tumor necrosis factor-alpha (TNFα) and interleukin-1 (IL-1)).

We suggest that one of the risk modifiers for IE may be the ST2L transmembrane receptor (suppression of tumorigenicity 2 ligand). The ST2L protein is a member of the Toll/interleukin-1 superfamily – highly conserved intracellular signaling domains. Representatives of this family initiate innate immunity by activating the transcription factor NF-kappa B (NF-kB), which leads to the formation of pro-inflammatory cytokines. However, it was found that ST2L forms a heterodimeric complex for binding of IL-33 to IL-1R. The IL-33/ST2 signaling complex can stimulate the immune responses of both type 1 helper T cells (Th1) and type 2 helper T cells (Th2) depending on the type of the activated cell, microenvironment, and cytokine network in the damaged tissue [3]. At the same time, in experimental works, it was shown that IL-33, which is a member of the IL-1 family, could independently function as a modulator of the NF-kB signaling pathway activity and the Toll-like receptor (TLR)/IL-1R canonical signaling pathway [4, 5].

The aim of the research was to collect available information on the relationship between the IL-33/ST2 system and the polymorphism of genes encoding its components, the changes in their expression level and the pathogenesis of infective endocarditis.

SEARCH STRATEGY

This review includes data from relevant articles describing the role of gene polymorphism of the innate immune response and the features of their expression in patients with infective endocarditis, published from January 2008 to January 2018 and presented in the PubMed database. Search queries were formulated with the following word combinations: "infective endocarditis", "gene expression", "interleukin", "cardiovascular diseases", "protein secretion". Search for publications not found with these combinations was performed using references in relevant articles.

INFECTIVE ENDOCARDITIS

Infective endocarditis (IE) is one of the multifactorial diseases. It comes second among the causes of the development of acquired heart defects. The key point in the formation of pathological changes in the valvular apparatus is an infection, usually of a bacterial etiology, which affects valvular and subvalvular heart structures and has an acute or subacute course [6, 7]. Microbial colonization is possible in damaged areas of native heart valves, or prosthetic structures. There is a probability of colonization of intracardiac implants and foreign intravascular prosthetic materials used in a wide range of therapeutic surgical interventions for the correction of cardiovascular pathologies. The progress in conservative and surgical treatment, the emergence of new risk groups, and the formation of microorganism resistance to a wide range of antimicrobial agents have led to the emergence of new clinical manifestations of IE, which complicates timely diagnosis and worsens the prognosis of the disease. Infective endocarditis that is not associated with intravenous drug use is a disease that occurs in both men and women (however, the incidence of IE in men is three times higher) at any age (but the risks are higher for people over 50 years old) [8].

To date, more than 120 pathogens of infective endocarditis are known. The leading pathogens are gram-positive bacteria [2]. Most often, they are representatives of the Streptococcus (*Str. Viridans, Str. Bovis*, etc.), Staphylococcus (mainly *S. aureus, S. epidermidis*) and Enterococcus genera. In some cases, the pathogen may be fungi and bacteria from the HACEK group (Haemophilus, Actinobacillus, Cardiobacterium, Eikenella, and Kingella), other gram-negative bacilli, and sometimes cocci [9].

Normally, the endothelium of the valvular heart apparatus is resistant to bacterial colonization under conditions of periodic transient bacteremia [6]. For the development of IE not related to the intravenous administration of narcotic drugs, a number of independent factors are required. They include a change in the heart valve surface to obtain a suitable place for bacterial colonization; stable bacteremia, with a circulating pool of highly virulent microorganisms; creation of an infected mass by "burying" a proliferating microorganism in the protective matrix of fibrin and platelets, the presence of immunosuppressive states, including suppression of the immune response associated with stress (hypothermia, malnutrition and insufficient nutrition, chronic psycho-emotional stress); a genetic predisposition due to mutational variability of genes of different protein classes [8].

At the site of attachment of the bacterial colony, an inflammatory reaction can be expressed up to the level of abscess formation with the subsequent valve leaflet destruction. The formation of abscesses is a significant complication in IE, since abscesses can penetrate deeper into the fibrous rings and myocardium [10]. In addition to the deformation of the leaflet apparatus, spreading on the heart valve prosthesis leads to formation of fistulas, which can cause a complete separation of the prosthesis from the fibrous ring. The atrial surface of the mitral valve leaflets and the ven-

tricular surface of the aortic valve leaflets are places of an increased risk of vegetation attachment, as they are areas of high pressure.

The inflammatory response in the development of IE is systemic and stimulates the development of reactions of both an innate and adaptive immune response, starting with acute-phase proteins, complement system activation, increasing concentrations of circulating immunoglobulins of all classes, the appearance of macrophages in peripheral blood, and the synthesis of various types of circulating antibodies [11]. To control the growing infectious lesion, the host organism intensively produces opsonic antibodies, cryoglobulins, antibodies against bacterial heat shock proteins and macroglobulins, and complement-fixing and agglutinating antibodies. Antibodies against cell surface components reduce the adhesion of *C. albicans* to fibrin and platelets *in vitro* and reduce the incidence of IE *in vivo*. Recent data indicate the possible role of vaccination against the clumping factor A for the IE prevention in simulation studies [12]. However, an effective vaccine for humans has not been developed yet.

Thus, the pathogenesis of IE includes several factors: a pathogenic microorganism, damage to the surface of the native heart valve or the presence of a prosthetic heart valve, the activity of the immune response, and exogenous and endogenous factors, including individual susceptibility to infection [13].

Even though the clinical component of IE has been studied sufficiently, opinions on the etiology of this disease are split [13]. Besides, the trigger mechanism for the development of the pathological process is not well understood from the perspective of the immune response activation. Information on the immune response reactions, marker genes, and their expression during the inflammatory response in IE is changing [15]. At the same time, some researchers [15] demonstrate the correlation between gene polymorphism of the innate immune response and susceptibility to *S. aureus* invasion and an increased risk of native valve endocarditis development.

INTERLEUKIN-33 (IL-33)

Interleukin-33 (IL-33), a member of the family of pro-inflammatory cytokines (IL-1), enters the cytoplasm and extracellular space when the cell is damaged. IL-33 is secreted in endothelial cells, epithelial cells, and fibroblasts, both during homeostasis control and in inflammation. IL-33 acts as an alarm (alarmin activity) and is released upon cell destruction or tissue damage to initiate immune cells [31]. IL-33 initiates and activates local inflammatory reactions by recruiting and activating cells that have functions associated with inflammation (eosinophils, basophils, and neutrophils), stimulates fibrogenesis and angiogenesis, affects vascular permeability (*in vitro* and *in vivo* models), and participates in the restoration of the mucous membrane integrity and wound healing [15].

The IL-33 gene is located on the 9p24.1 chromosome, with a length of 42,835 bases; it interacts with IL1RL1, USP21, GATA3, and other genes. The constant expression of IL-33 is registered in various types of epithelial cells, fibroblasts, smooth muscle cells, and mast cells [17]. The expression of IL-33 in macrophages is negligible, but its activation by anti-inflammatory factors, such as cell wall lipopolysaccharides, may appear [18]. A polymorphic variant of this gene with a mutation at rs7044343 is associated with the modulation of coronary heart disease [19].

Despite the fact that IL-33 belongs to the family of pro-inflammatory IL-1, the presence of immunoregulatory properties distinguishes it from IL-1 and the fibroblast growth factor, which obtained structural similarity to IL-33 [20]. Being an important member of the IL-1 family, IL-33 has pleiotropic effects in the formation of the innate and adaptive immune responses, and its level strictly correlates with the level of inflammation in the tissue [21]. At the same time, IL-33 can regulate the nuclear transcription of protein genes, which activate inflammation. IL-33 acts as a traditional cytokine by activation of NF-kB via the ST2L/IL-1RAcP dimeric complex, or as an intracellular activator of a nuclear factor by translocation into the nucleus, where it binds to chromatin and modulates gene expression. IL-33 can act as an alarm when it is released after cell damage or as a negative regulator of NF-kB gene transcription when it acts intracellularly [3]. IL-33 is synthesized in the form of a precursor with a molecular mass of 30 kDa. After propeptide cleavage influenced by the caspase 1 enzyme, it is transformed into a mature protein with a mass of 18 kDa. The precursor form is processed enzymatically and then initiates inflammation through the Toll-like receptor (TLRs) recognizing signaling system, acting as an alarmin [22]. However, synthesized IL-33 may not go through the maturation stage. In this case, it acts as a transcription inhibition factor due to the presence of a nuclear localization signal in the propeptide. The transcriptional repressor function, which is not characteristic of the cytokine family, is realized through bonds with the surface of the nucleosome in the pocket

region formed by histones H2A and H2B [20]. Also, IL-33 can serve as a non-histone chromosomal protein involved in the assembly of nucleoprotein complexes, supporting and strengthening the chromatin structure, which affects the gene expression rate in these chromosome regions. IL-33 is expressed by both immune cells, for example, macrophages and dendritic cells, and non-immune ones – endothelial and epithelial cells, and fibroblasts [21]. Unlike other members of the IL-1 family, IL-33 primarily induces Th2 immune responses and macrophage polarization through an alternative activation pathway (the so-called M2 macrophages) [10]. The release into the extracellular space occurs after tissue damage [23] and is accompanied by Th2 initiation and stimulation of the secretion of associated cytokines (IL-4, IL-5, and IL-13), as well as by activation of the innate immune response cells – mast cells and lymphoid cells of the innate immune response (innate lymphoid cells, ILCs).

To activate the NF-kB and MAPK via the MyD88-dependent signaling pathway, IL-33 binding to the plasma membrane receptor [3], consisting of ST2L receptor proteins (tumor suppressor ligand 2) and IL-1RAcP (interleukin 1 receptor auxiliary protein), is required. In addition to its role in the autoimmune response, which is the most studied [24], IL-33 is involved in the inflammatory processes accompanying various cancer, pulmonary, intestinal, and cardiovascular diseases [25]. There is also evidence of its role in the pathogenesis of Alzheimer's disease [26]. However, despite the obvious immunoregulatory effect on the course of IE, the participation of IL33 in the pathogenesis of this inflammatory disease is not described.

SUPPRESSION OF TUMORIGENICITY 2 (ST2)

The suppression of tumorigenicity receptor (ST2), also known as IL1RL1, T1, DER4, or Fit-1, is a member of the interleukin family. The IL1RL1 gene is located on the long arm of the 2q12 chromosome and contains 11 exons. IL1RL1 can perform the functions of an immunomodulator, therefore it is expressed both as a receptor attached to a membrane, activated by IL-33, and as a soluble variant (sST2), which exhibits anti-inflammatory properties.

As a result of alternative splicing of IL1RL1, ST2 can be expressed in four functional isoforms: ST2L (membrane-bound form), sST2 (soluble form), ST2V – an isoform similar to sST2 but lacking the third extracellular domain of immunoglobulin [27], and ST2VL with a transmembrane domain. ST2L is expressed by various immune cells, such as mast cells, monocytes, dendritic cells, and is selectively expressed on Th2 cells, but not on Th1 lymphocytes [28].

In addition to the expression of ST2L on the surface of many immune cells (lymphocytes (Th2), natural killer (NK) cells), the expression of this receptor on the surface of myeloid cells, such as monocytes, dendritic cells and granulocytes, was identified [3]. It was found that the effects of ST2L are realized through the negative control of IL-1RI and TLR4, which consists in blocking the MyD88 and Mal adaptor proteins. This blocking leads to the inhibition of TLR signaling and promotes the development of a Th2 immune response. However, the control does not extend to the TLR3 signaling pathways and does not affect the transcription factor of type III interferons (IFNs), which makes it possible to further regulate the inflammatory response to virus infection through activation of interferon alpha and beta (IFNα, -β) transcription, as well as through other interferon-induced genes.

At the same time, sST2 binding to IL-33 leads to blocking of the signaling pathway along the IL-33/ST2L axis [30] and reduces anti-inflammatory effects, thus eliminating the cardioprotective effect. It is believed that the soluble form of ST2, as an IL-33 trap, plays a crucial role in several autoimmune diseases, including systemic lupus erythematosus, sclerosis, and rheumatoid arthritis [30, 32]. Being a mechanically induced cardiomyocyte protein, sST2, depending on its level in the serum, can predict the outcome in patients with acute myocardial infarction or chronic heart failure. The sST2 can also disrupt cardiac function and exacerbate heart remodeling in both non-ischemic and ischemic tissues. In addition to the role of IL-33/ST2L as a therapeutic target, sST2 has also been identified as a biomarker of ischemic heart disease in humans [33]. It was shown that the introduction of the sST2-Fc fusion protein can be useful in the treatment of arthritis, pulmonary eosinophilia, shock, liver, and intestinal ischemic reperfusion injury [34, 35]. It was found that sST2 blocks the production of pro-inflammatory cytokines, such as IL-6, IL-12, and tumor necrosis factor-alpha (TNFα) by macrophages, caused by lipopolysaccharides (LPS) of the cell walls of gram-negative bacteria, but does not affect the production of IL-10. It was also revealed that genetic variants that change the intracellular transmembrane signaling of ST2 can express human sST2, opening a new pathway of immune and inflammatory regulation [36].

Thus, it has been proven that ST2 can regulate the inflammatory response to tissue damage mainly by modulating signaling of the MyD88 and Mal adaptor receptors, which is directly related to the nuclear factor NF-kB from the TLR activation pathway. TLRs are the main receptors of the innate immune response that recognize elements of the bacterial cell walls and associated mechanisms of inflammation activation in response to bacterial invasion, including the formation of conditions for valve structure colonization by the opportunistic *Streptococcus bovis/Streptococcus equinus* complex associated with the immune evasion [37]. Due to this fact, the participation of the ST2 protein in the pathogenesis of IE seems logical. However, this direction has not acquired sufficient attention.

RECEPTOR COMPLEX IL-33/ST2

As mentioned earlier, one of the factors of the NF-kB pathway activation which is accompanied by stimulation of Th1 or Th2 immune responses, depending on the type of the activated cell, microenvironment, and cytokine content in the damaged tissue, includes the IL-33/ST2L complex [3]. In experimental models of one study, it was found that the development of type 1 diabetes, experimental autoimmune encephalomyelitis, fulminant hepatitis, and breast cancer was accompanied mainly by the Th1/Th17 immune response. At the same time, a higher level of IL-33 production was recorded [3]. M. Milovanovic et al. suggested that IL-33, in a manner independent of its receptors, may contribute to the development of inflammatory autoreactive immune responses.

It is noteworthy that the receptor for IL-33 is a heterodimeric complex consisting of the membrane-bound ST2L protein and the IL-1RAcP co-receptor. The basis of the effector initiation of Th2-type responses is the activation of the ST2L receptor, which, despite its membership in the TIR family, is not involved in the activation of NF-kB. The initiation of inflammatory reactions through the IL-33/ST2 receptor complex occurs due to the signal-conducting co-receptor IL-1RAcP and depends on the type of cell and microenvironment. Various signaling pathways activated by IL-33, including MyD88, IL-1R-associated kinase 4 (IRAK4), and TRAF6, have been described [38]. With the use of the downward pathway through CT2, MyD88, and TRAF6 adapters, activation of NF-kB and mitogen-activated protein kinases, which are involved in the control of cell proliferation and apoptosis, is ultimately possible [38]. Members of the mTOR pathway, such as phosphoinositide-3-kinase (PI3K), can also be activated by IL-33 in Th2 cells, macrophages, or eosinophils [39], which positively correlates with the expression of IL-33 and sST2 genes in myocardial infarction [40].

The IL33/ST2L signaling pathway is also regulated by other mechanisms, for example, IL-1RAcP alternative splicing, and affects the severity of the inflammatory response, since signaling depends on the functional state of both components [41]. IL-1RAcP is also important for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinase (SAPK). The recombinant chimeric sIL-1RAcP-Fc protein has been found to decrease IL-6 secretion in mast cells exposed to IL-33 [41]. K. Hong et al. showed that co-incubation of sST2-Fc and sIL-1RAcP-Fc synergistically inhibited the activity of IL-33, indicating the role of sIL-1RAcP in modulating the biological activity of IL-33.

At the same time, the ST2 ligand-binding chain with the component for interacting with IL-33 and the soluble ST2 have antagonistic properties. This mechanism allows to regulate the inflammatory response activation in various types of cells and tissues. However, it is assumed that an additional, different from IL-1RAcP and ST2, receptor component that can participate in the regulation of the biological activity of IL-33 exists [41]. It is suggested that such a component involved in the activation of the signaling pathway is an Ig IL-1R-related molecule (SIGIRR) interacting with IL-1RR (SIGIRR), a member of the IL-1R family, which is involved in the regulation of IL-18, IL-1, and IL-33 signaling. In Th-2 cells exposed to IL-33, dimerization of SIGIRR with ST2L negatively regulates IL-33/ST2 signaling through direct interaction with the intermediate signaling block of the IL-1R family. The IL-1R and IL1RAcP complex blocks downward signaling through the extracellular domain of IL-33 or by its interaction with MyD88, IRAK, and TRAF6 [42].

THE ROLE OF THE IL-33/ST2 COMPLEX IN THE INFLAMMATORY RESPONSE

As described above, the IL-33/ST2 complex can have both pro-inflammatory and anti-inflammatory effects. Elevated levels of soluble ST2 may indicate a risk of developing graft-versus-host disease (GVHD) and its further mortality. Elevated levels of IL-33 in mice after conditioning and in patients during GVHD were shown in a study by D.K. Reichenbach et al. [43]. IL-33/ST2 activation was performed on murine and human alloreactive T cells. It was shown that

sST2 concentration increased as experimental GVHD progressed. Blocking IL-33/ST2 interactions during transplantation of allogeneic hematopoietic cells by exogenous ST2-Fc infusions was characterized by a decrease in mortality during GVHD. This fact indicates that ST2 acts as a trap receptor that modulates GVHD.

Studies have also shown the important role of IL-33 and ST2 in the inflammatory processes of the respiratory system. For example, IL-33 level correlates with the severity of clinical asthma [43], since IL-33 increases the level of type 2 cytokines that mobilize eosinophils and polarize M2 macrophages. Probably, the same mechanism is present in an increase in the IL-33 concentration in patients with atopic dermatitis who have quite high IL-33 level in the skin epidermis [44].

Endometriosis is a chronic condition that is classified by the abnormal growth of endometrial tissue outside the uterus. Although the pathogenesis of this disease remains unknown, it is noted that patients with endometriosis have immune dysfunction. J.E. Miller et al. [45] investigated the role of IL-33 as a regulator of chronic inflammation, which plays a critical role in the pathology of endometriosis, using patient tissue samples, cell lines, and a syngeneic mouse model. It was found that in the tissue with endometriosis, significantly higher levels of IL-33 protein are observed compared to the endometrium of healthy fertile organs. In vitro stimulation of IL-33 led to the production of pro-inflammatory and angiogenic cytokines. In the syngeneic mouse model of endometriosis, injections of IL-33 caused systemic inflammation, resulting in an increase in pro-inflammatory plasma cytokines compared to the control group. In addition, endometriotic lesions in IL-33 treated mice were highly vascularized, while the cells involved in this process showed increased proliferation. The authors provided strong evidence that IL-33 stimulates inflammation, angiogenesis, and proliferation in the endometrium.

Cigarette smoke causes lung epithelial cells to produce intracellular IL-33 more intensively, which is released after cell damage by a viral or bacterial infection. At the same time, the production of ST2 by congenital type 2 cells is reduced, but its expression by macrophages and NK cells is increased, which leads to a halt in the production of type 2 cytokines by ILC2 and inhibition of IL-12 production by macrophages [46].

It has been shown that the expression of IL-33 and ST2 increases in the gingival tissue in patients with chronic periodontitis and COPD, which makes them potential therapeutic targets. In contrast, IL-33 plays an important role in uveitis, which is an auto-inflammatory disease affecting the eyes. Treatment with IL-33 medications reduced the severity of experimental autoimmune uveitis in mice, thus, suggesting the possibility of using recombinant IL-33 to treat autoimmune uveitis and autoimmune diseases in general [47].

In recent years, knowledge about the role of IL-33, sST2, and the IL-33/ST2 complex in the pathophysiology of cardiovascular diseases has expanded. Data on the association of these proteins with dysfunction, fibrosis, and myocardial remodeling have appeared. In this regard, the beneficial effects of IL-33 are realized through the ST2L receptor. When IL-33 binds to sST2, the interaction between ST2L is interrupted and antiremodeling effects are eliminated [48].

Besides its role in myocardial remodeling, the IL-33/ST2 system, presumably, plays an additional role in the development and progression of atherosclerosis. It is suggested that the IL-33/ST2L complex may have therapeutic potential for the beneficial regulation of myocardial response to overload and trauma [47]. It was shown that after MI, sST2 expression increases rapidly during the first 4 weeks and, unlike IL-33, its levels correlate with the processes of fibrosis and inflammation. The obtained data indicate the differential regulation of IL33 and sST2. The therapeutic modulation of early sST2 expression may be more important to prevent unfavorable remodeling after MI [49].

One part of the Framingham study showed that elevated serum sST2 levels are associated with genetic determinants and correlate with an increased risk of cardiovascular diseases [50]. With the participation of 2,991 individuals, the authors were able to establish that differences in sST2 levels are caused to a greater extent by genetic factors than by clinical and environmental ones. The GWAS (genome-wide association study) demonstrated multiple associations between single nucleotide polymorphism (SNP) in different parts of the IL1RL1 gene and sST2 concentrations. Five missense mutation variants of IL1RL1 showed a correlation with higher levels of sST2 and were found in exons encoding the intracellular domain of ST2, which is absent in sST2. The authors show that the genetic variation of IL1RL1 can lead to an increase in sST2 levels and alter immune and inflammatory signaling via the ST2/IL-33 pathway.

In a study involving individuals in the Chinese Han population, a relationship between polymorphisms of the IL-33/ST2 signaling pathway and MI was found [51]. An analysis of the case-control study with the par-

ticipation of 490 patients with MI and 929 individuals in the control group was carried out. The relationship between the polymorphic variants of IL33, IL1RL1, and IL1RaP (rs11792633, rs1041973, rs4624606) and the risks of developing MI was studied. Based on an associative study, it was concluded that in the IL33/ST2 signaling pathway, the minor allele of polymorphism rs4624606 IL-1RaP is a potential independent risk factor for MI.

The role of IL33 as an alarmin in activating the dependent Th2-type response in the development of obesity, viral infections, immunological deficiency, intestinal inflammation, suppression of tumor growth [52], and cytomegalovirus infection has been demonstrated [53].

Thus, many authors have shown that the IL33/ST2 immune receptor complex is one of the first to be included in pathological changes, both in diseases associated with tissue damage and in response to microbial invasions. The functioning of the complex consists not only in the activation of the innate immune response along the effector pathway but also in the development of an inflammatory response along the Th2 activation pathway. The latter is accompanied by a decrease in the local inflammatory response, which may be associated with an increased risk of microbial adhesion on the valvular heart apparatus and the development of infective endocarditis.

Since IL-33/ST2 activation effects are multidirectional and depend on many external factors, including tissue and microenvironment, its role in modulating the inflammatory response is undeniable. Currently, insufficient attention is paid to the role of the IL-33/ST2 signaling complex in infectious pathologies of bacterial nature.

EXPRESSION OF MRNA AND IL-33/ST2 PROTEINS IN CARDIOVASCULAR DISEASES

Even at early stages of studying the immune response through the IL33/ST2 system [54], using the real-time polymerase chain reaction (real-time PCR) method, it was found that the IL1RL1 gene is actively expressed in hematopoietic cell lines. It was also actively expressed in the helper lines of T cells in the lymphocyte culture lines. It was found that mouse cell lines with Th1 lymphocytes do not express ST2 mRNA. On the other hand, one of the Th2 cell lines, D10, expressed ST2L (transmembrane form) without stimulation, while co-stimulation of PMA and A23187 induced ST2 (soluble form) of mRNA. These results indicate that the ST2 gene is involved in the regulation of the immune system. IL-1α, IL-1β, and the receptor antagonist did not bind to the ST2L protein, which prompted the authors to search for a specific ST2 ligand. In the experiment, the recombinant human ST2 protein was purified and labeled with FITC. As a result, labeled human ST2 bound to RPMI8226 cells derived from myeloma was detected among various B cell lines, indicating the possible involvement of ST2 in T cell/B cell interaction.

Inflammatory cytokines, including IL-33/ST2, are involved in the regulation of adaptive and non-adaptive changes in the heart. Since cardiac fibrosis is largely dependent on increased production of extracellular matrix by cardiac fibroblasts, J. Zhu et al. [55] suggested that IL-33 inhibits the pro-fibrous activity of these cells directly. However, the concentration of IL-33 did not affect the expression of genes encoding the components of the extracellular matrix, or proliferation (typical of fibrosis markers). In a simulation study in mice, it was demonstrated that IL-33 is predominantly produced by myocardial fibroblasts, rather than by cardiac myocytes. This study showed that when knocking out the ST2 gene, mice are more susceptible to TAC-induced cardiac hypertrophy. Another study by D. Shao [56] showed that knocking out IL-33 disrupts the active system that protects the myocardium from cardiac remodeling events, such as cardiomyocyte hypertrophy and cardiac fibrosis induced by mechanical stress.

Modulation of the IL-33/ST2 system in post-infarction heart failure in rats showed increased levels of mRNA expression during myocardial infarction for IL-33 and sST2, but their different kinetics [49]. IL-33 mRNA expression was high immediately after acute myocardial infarction (AMI) and remained elevated for the first 12 weeks after AMI, which was accompanied by an increase in IL-33 protein expression. In contrast, sST2 mRNA expression showed an early peak 1 week after AMI, followed by a dramatic decrease in the first 4 weeks. Although sST2 expression showed an early peak and a positive correlation with markers of fibrosis and inflammation, IL-33 expression levels remained high over the entire observation time and did not correlate with these markers [48]. Cultivating human primary cardiac fibroblasts and human primary cardiac myocytes, P.T. Veeraveedu et al. [57] measured the mRNA, IL-33, and ST2 protein expression levels in cells and examined the effect of cytokines on the expression of these genes. Pro-inflammatory cytokines increase the expression of IL-33 in cardiac fibroblasts of the heart, cardiac myocytes, and vascular

smooth muscle cells through the pathways of NF-kB and MEK, which proves its participation in inflammatory processes of the cardiovascular system. Besides, it has been shown that IL-33 is released during necrosis of human cardiac and smooth muscle cells.

In *in vivo* experiments, knockdown of IL-33 in normal endothelial cells of the human pulmonary artery led to the induction and expression of sST2. This is associated with the action of IL-33 as a nuclear suppressor for reducing sST2 expression by binding to homeobox regions and potentially recruiting transcriptional repressor proteins. As a result, a study by D. Shao et al. [56] showed that a significant loss of IL-33 occurs without its release from the cells in idiopathic pulmonary arterial hypertension.

Therefore, IL-33 enhances immune responses and inflammation, depending on the reactions of the immune response cells. On the other hand, the effects of IL-33 are associated with the induction of the Th2-type immune response through its ST-2 receptor. IL-33 is also a nuclear repressor factor. ST2, in turn, is expressed both as a variant of the ST2-membrane-dependent receptor activated by IL-33 and as a soluble sST2 variant, which manifests itself as a receptor trap and has anti-inflammatory properties. Despite the studied participation of the IL33/ST2 complex in many pathological processes, including asthma, rheumatoid arthritis, inflammatory bowel diseases, and more recently, cancer, Alzheimer's disease, cognitive disorders, and malaria [58], the involvement of this complex in the inflammatory response in infectious diseases is not clear.

CONCLUSION

The accumulated data on the participation of IL-33, sST2, and the IL-33/ST2 receptor complex in inflammatory and cardiovascular diseases prove the significant role of these cytokines in the inflammatory response in tissue damage and viral infection. However, the question of their participation in the pathogenesis of infective endocarditis is neglected. Like all cytokines, IL-33 and ST2 function more actively at low concentrations, and, therefore, their functionality remains unknown when their concentration changes during IE, which is inevitably accompanied by a change in local cytokine status and destruction of valve tissue. The problem of a quantitative assessment of gene expression and protein secretion of these interleukins in case of heart valve infection remains relevant.

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Biogenic polyamines and genital gonococcal infection: facts and hypotheses

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ABSTRACT

Genital gonorrhea is one of the most common sexually transmitted diseases with significant gender differences in its clinical course. Laboratory verification of the diagnosis is associated with great difficulties in the cultivation and identification of the pathogen. Moreover, the diagnosis of female gonorrhea is a serious problem due to mild symptoms of the disease. Currently, a promising trend in the diagnosis of inflammatory diseases of reproductive organs is biochemical analysis of vaginal and sperm fluids, which have a rich component composition. Biogenic polyamines can be synthesized by both pro- and eukaryotic cells. These polycations are present in semen and vaginal fluid and can have a significant effect on various cell structures and functions. In this regard, the qualitative and quantitative composition, the level and ratio of these components and their changes can have a diagnostic value for infections of the genital tract.

The aim of the review was to analyze current information on the role of biogenic polyamines in the physiological and biochemical potential of *Neisseria gonorrhoeae* and their participation in the development of genital gonococcal infection, taking into account the influence of sexual differences and a number of related factors. Special attention was paid to the origin and possible functional role of polyamines in the genital tract of men and women. As a result, taking into account the spectrum, origin and ratio of polyamines in the corresponding fluids, we formulated a hypothesis: the manifestation of the process in case of infection in men is largely determined by the reactivity of eukaryotic cells, but not the metabolic activity of the microbiota of the reproductive tract. At the same time, the development of "female" gonorrhea is primarily determined by the state of the microbiocenosis of the cervical vaginal biotope.

Key words: genital gonococcal infection, *Neisseria gonorrhoeae*, biogenic polyamines, microbiota, biofilms, antibiotic resistance.

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Биогенные полиамины при генитальной гонококковой инфекции: факты и гипотезы

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РЕЗЮМЕ

Генитальная гонорея является одним из наиболее распространенных венерических заболеваний и характеризуется существенными гендерными различиями в его клиническом течении. Лабораторное подтверждение диагноза сопряжено с большими сложностями культивирования и идентификации возбудителя, а диагностика «женской» гонореи представляет серьезную проблему еще и в связи со стертой симптоматикой инфекционного процесса. На современном этапе перспективным направлением для диагностики воспалительных заболеваний репродуктивных органов признается изучение биохимического состава влагалищной и спермальной жидкостей, имеющих богатейший компонентный состав. Биогенные полиамины, которые могут синтезироваться как про-, так и эукариотическими клетками и в значительных количествах обнаруживаться в этих секретах, являются низкомолекулярными соединениями, оказывающими разнообразные эффекты на жизненно важные структуры и функции клеток обоих типов. В этой связи качественный и количественный состав, уровень и соотношение этих компонентов в секретах, с учетом изменения соответствующих показателей в динамике, могут иметь диагностический смысл при инфекционной патологии генитального тракта. Целью обзора явилось рассмотрение накопленной к настоящему времени информации о возможной роли биогенных полиаминов в физиолого-биохимическом потенциале *Neisseria gonorrhoeae* и их участии в развитии генитальной гонококковой инфекции с учетом влияния половых различий и ряда сопутствующих факторов. Особое внимание уделено происхождению и возможной функциональной роли полиаминов в генитальном тракте мужчин и женщин. В результате, с учетом спектра, происхождения и соотношения полиаминов, доминирующих в составе соответствующих секретов, сформулирована гипотеза о том, что манифестация процесса в случае инфицирования мужчин в большей степени обусловлена реактивностью эукариотических клеток, но не метаболической активностью микробиоты их репродуктивного тракта. В то время как развитие «женской» гонореи в первую очередь определяет состояние микробиоценоза цервикально-вагинального биотопа.

Ключевые слова: генитальная гонококковая инфекция, *Neisseria gonorrhoeae*, биогенные полиамины, микробиота, биопленки, антибиотикорезистентность.

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

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INTRODUCTION

Gonococcal infection (GI) and its most common form, acute genital gonorrhea, is an infectious disease transmitted *primarily by sexual contact* and caused by *Neisseria gonorrhoeae* [1, 2]. Laboratory verification of the diagnosis is associated with difficulties in the cultivation and identification of the pathogen. Moreover, the diagnosis of "female" gonorrhea is a serious problem due to mild symptoms of the infectious process. Many researchers note that the promising trend in the diagno-

sis of inflammatory diseases of reproductive organs is the study of seminal and vaginal fluids [3–6], since they have a complicated biochemical composition comparable to blood. Even if we take into account only proteins with a molecular mass of 10–100 kDa, more than 200 of them were known in the sperm plasma of healthy fertile men in 1981 [7]. However, this huge diagnostic potential is used only to a small extent due to the lack of data on the physiological role of various components of these fluids in inflammation of the reproductive organs [4, 8–10]. Recently, a growing interest has appeared in biogenic polyamines (BPA) and their level, since these compounds are present in all pro- and eukaryotic cells and perform various functions, including protective ones. The content and balance of BPA change dynamically, which may be useful for diagnosis. The information about the involvement of BPA in the persistence of *N. gonorrhoeae* and development of genital GI is presented in a few studies and is often contradictory.

The aim of this article was to review the current information on the role of BPA in the physiological and biochemical potential of *N. gonorrhoeae* and their possible participation in the development of genital gonococcal infection, including the influence of gender differences and a number of related factors.

CHEMICAL STRUCTURE, SYNTHESIS AND TRANSPORT OF BPA

Biogenic polyamines are aliphatic hydrocarbons possessing two or more amino or imino groups and differing in the length of the carbon chain. The most widespread polyamines in living organisms are diamines: putrescine, cadaverine; triamines: spermidine, norspermidine; and tetraamines: agmatine and spermine [11].

Eukaryotic cells contain mainly spermidine and spermine, whereas putrescine is found in trace amounts. These polyamines are widely represented in various organs, tissues, and biological fluids of the human body both as free forms and as complexes with proteins and nucleic acids. The highest concentrations (millimoles) of spermidine and spermine are found in the sperm plasma of men [12], while in the vaginal discharge of women polyamines are detected in micromolar amounts [13]. On the contrary, the concentrations of putrescine and cadaverine in prokaryotic cells is the highest, spermidine is present in small amounts and spermine is practically not detected [11, 14, 15]. Some bacteria are capable of producing diaminopropane, homospermidine or norspermidine [11, 16]. The spectrum and quantity of BPA are significantly different in various species of microorganisms. Based on this fact, some authors have attempted to use polyamines as chemotaxonomic markers [16–18]. However, this approach does not seem to be promising, since the polyamine pool in the microbial cells depends on the age of the culture and growth conditions, such as composition and pH of the medium, temperature, aeration, etc. [19–21].

The intracellular content of polyamines is determined by several parameters: biosynthesis level, activity of degradation, as well as the intensity of the exchange of these components between the cell and the environment. Genes involved in the polyamine biosynthesis are found in genomes of many microorganisms; however, the active *de novo* synthesis of these compounds is mostly typical of gram-negative bacteria [22–24]. Putrescine can be synthesized in several ways: directly from ornithine by ornithine decarboxylase or from arginine by arginine decarboxylase with the formation of agmatine, or via N-carbamoyl putrescine [25, 26]. Spermidine is synthesized on the basis of putrescine and S-adenosylmethionine using the enzymes S-adenosylmethionine decarboxylase, aminopropyl transferase and spermidine synthetase [27, 28], or from putrescine and aspartate semialdehyde with involvement of carboxyspermidine decarboxylase and carboxyspermidine carboxylase [29–31]. Cadaverine synthesis in bacteria is realized by direct decarboxylation of lysine with lysine decarboxylase [32]. Among all the known polyamine synthesis enzymes, only arginine decarboxylase, that catalyzes the synthesis of agmatine (precursor of putrescine) from arginine, was detected in *N. gonorrhoeae* [33]. There are no data on the activity of this enzyme in *N. gonorrhoeae* in the available literature, which is probably determined by the difficulties in cultivating this microorganism under laboratory conditions and obtaining a sufficient amount of biomass.

The absence of other polyamine synthesis enzymes in gonococci can be partly explained by their specific habitats. In the urogenital tract of men, polyamines are present in large amount [12, 34], and bacteria can receive these compounds via transport. Many gram-negative bacteria are capable of transporting polyamines from the environment. For example, four BPA transport systems are found in *Escherichia coli* cells. Two of them (spermidine-preferential system PotDABC and putrescine-specific system PotFGHI) are ABC (ATP binding cassette) transporters [35]. Each of them consists of a periplasmic substrate-binding protein (PotD and PotF), two channel-forming transmembrane proteins (PotBC and PotHI) and membrane-associated ATPase (PotA and PotG) [36, 37]. The third transport system (PotE) catalyzes the uptake and excretion of putrescine [38]. Cadaverine is transported via the lysine-cadaverine antiporter CadB [39]. In contrast to enterobacteria, the transport of polyamines in gonococci is not sufficiently studied. The PotFGHI transport system was found in *N. gonorrhoeae*. It is similar to *E. coli* transport system and selectively

transports spermine and spermidine, but not putrescine and cadaverine into the cell from the medium [40]. There is evidence that the agmatine/arginine antiport is encoded in the gonococcal genome. This system provides the uptake of agmatine in exchange for arginine, which is important for survival of *N. gonorrhoeae* in the acidic environment [33].

PARTICIPATION OF POLYAMINES IN PHYSIOLOGICAL PROCESSES

The fact that intracellular pool of BPA in many microorganisms reaches quite high (millimolar) concentrations even with growth on minimal media indicates the importance of these compounds for the cell physiology. Participation of polyamines in various cell processes is a consequence of their chemical structure. Under physiological conditions, amino groups in polyamine molecules are protonated and, thus, positively charged. Thereby, BPA are able to interact with negatively charged cell components, such as DNA, RNA, proteins [41, 42], membrane phospholipids, and cell wall structures [43]. Polyamines can modulate conformation and maintain structural and functional stability of the cell components, also indirectly through transport processes. It is shown that the concentration of BPA in the cell envelope changes in response to external influences that affect the porin activity and thus regulate the permeability of the outer membrane [44–46]. BPA are known to be involved in regulating nucleic acid synthesis, in particular, the replication process [27], as well as in maintaining the conformation of DNA and RNA [47, 48]. Of great importance is their ability to affect gene expression at the stages of transcription and translation [37, 49]. A group of genes which expression is regulated by polyamines at a translation level is called a "polyamine modulon" [50]. There is evidence that polyamines can affect the phosphorylation of specific proteins as well as modulate their degradation [49, 51].

The fact that polyamines are necessary for growth of *Neisseria* was first mentioned in 1952 [52]. It was later shown that polyamines along with other cations stabilized *N. gonorrhoeae* cells, preventing lysis. The same authors suggested the participation of BPA in the cell division process [53].

PARTICIPATION OF BPA IN ADHESION, BIOFILM FORMATION AND AGGREGATION OF BACTERIA

A lot of studies in recent years have indicated that BPA can be involved in the regulation of microbial adhesion, biofilm formation and aggregation [29, 54]. There is growing evidence that polyamines are able to influence biofilm formation by various commensal and pathogenic bacteria, including *E. coli, Bacillus subtilis, Staphylococcus aureus, Yersinia pestis, Enterococcus faecalis, Vibrio cholerae*, etc. [42, 55–57]. It is shown that not only polyamines of a host organism can regulate this process, but also the bacterial BPA. In particular, norspermidine, synthesized exclusively by bacteria, can inhibit the biofilm formation. However, the effect of BPA depends on the type of bacteria. For example, norspermidine effectively blocks the formation of a biofilm of *B. subtilis* and other bacterial species in laboratory conditions, and can cause its dispersion due to disruption of the matrix structure [58], but does not inhibit the formation of gonococcal biofilms and does not cause their dispersion [60].

N. gonorrhoeae cells in sperm, containing high concentrations of polyamines, must be adapted to such a medium. It is experimentally shown that seminal plasma and spermine inhibit adhesion of gonococci, but promote the aggregation of bacteria and formation of microcolonies [59]. A number of researchers indicate that spermine promotes the biofilm formation [60, 61]. These seemingly contradictory effects may be significant for successful colonization of biotopes and distribution of *N. gonorrhoeae*. Disruption of the contact of fixed cells with the surface can be important in the transmission of infection, in particular, when bacteria colonizing the male urethra leave it under the influence of sperm, providing their transition to the sexual partner [59].

However, it is possible that such results are determined by the specific methodological approaches to study the effect of seminal plasma. Biofilm formation is usually studied in a static system on a polystyrene surface by the method proposed at the end of the last century [62], while the adhesion of *N. gonorrhoeae* is studied using glasses or glass flow chambers [61]. On the other hand, it was shown that the specific twitching of *N. gonorrhoeae*, which is facilitated in the presence of seminal fluid, depends on the fluctuation of PilT pili. Despite the fact that pili are necessary for adhesion, the interruption of this process in the presence of spermine was not dependent on the presence of pili. Aggregation of gonococci in seminal plasma was also stimulated independently of these surface structures, which was demonstrated using mutants without pili [61]. It is assumed that BPA can greatly affect the ability of gonococci to form biofilms not only in the male, but also in the female urogenital tract due to a significant increase in spermidine and spermine concentrations after copulation with up to 15 mM spermine. [63].

It is believed that polyamines contribute to the transition of bacteria to the biofilm formation due to their toxic effects. This opinion is supported by the fact that

spermine has the highest effect on this process among the four biogenic polyamines (putrescine, cadaverine, spermidine and spermine); at the same time it is the most toxic in high concentrations, especially in relation to gram-positive microorganisms [64, 65] . Meanwhile, it was experimentally shown that polyamines did not affect the viability of planktonic gonococci in physiological concentrations. The mutants with a damaged spermine and spermidine transport system (potHI) demonstrated that the transport of polyamines did not affect the biofilm formation. The authors of this study believe that, most likely, the key point is the effect of BPA on surface structures and bacterial aggregation of *N. gonorrhoeae* [40].

A study of the three-dimensional structure of *N. gonorrhoeae* biofilms showed that atypical films formed in the presence of spermine. They contained fewer cells and had a more condensed matrix, in which the channels providing the influx of nutrients and oxygen as well as excretion of metabolic products of bacteria were hardly visualized [40, 66]. Such biofilms, on the one hand, may be less viable; however, on the other hand, slow metabolism due to deficiency of nutrients can contribute to the development of persistent forms. At the same time, a study of the polyamine influence on mature biofilms showed that none of these compounds had an effect on their dispersion [60]. It should be noted that laboratory methods for assessing the ability of microorganisms to form biofilms, especially in the case of hardly cultivated bacteria, often do not take into account various aspects of the influence of environmental conditions that exist *in vivo* (pH, the presence of various substances in the medium, etc.). It can also be the cause of contradictory research results.

PROTECTIVE ROLE OF BPA IN THE GENITAL SECRETIONS

It is known that bacteria are able to synthesize BPA constitutively under normal conditions; however, many microorganisms activate the polyamine synthesis in response to environmental influences [21, 67, 68]. These studies were published at the end of the past century and focused on the fact that BPA perform protective functions. Polyamines are involved in adaptation to various types of starvation, heat and osmotic shock, oxidative stress, pH-shifts, and other stresses [20, 69, 70]. One of the most common types of negative effects for *N. gonorrhoeae* is an acidic environment. Lactic acid in the vaginal fluids of women protects against pathogenic microorganisms [71, 72]. The role of polyamines in the adaptation of *N. gonorrhoeae* to low pH values was first demonstrated as far back as 1976 [73]. Recent studies have shown that putrescine and cadaverine increased the survival of gonococci in the presence of lactic acid, stabilizing the cell wall and membrane [33]. These diamines are mainly produced by microorganisms and are actively formed in females with vaginosis [74]. Under these conditions, the chance of developing GI can increase greatly.

However, not only polyamines, but also their precursors are able to protect bacteria from the effect of acid. It was experimentally shown that arginine, glutamate, and lysine increase the resistance of *E. coli* and other bacteria to the lethal effect of low pH [75]. Only arginine and agmatine, but not glutamate or lysine, increase the acid resistance of gonococci [33]. The difference in the effects of amino acids may be explained by the fact that only one polyamine synthesis enzyme – arginine decarboxylase – is present in *N. gonorrhoeae* cells, which activity with the formation of agmatine is accompanied by a decrease in the medium acidity. Agmatine-arginine antiport promotes the exit of agmatine into the medium in exchange for arginine, providing resistance to the action of acid [71]. An increase in acid resistance under the arginine action is of practical importance, since a large amount of this amino acid is contained in seminal fluid (7.3 ± 1.5 mM) [76]. It is believed that after sexual intercourse, when genital secretions are mixed, the concentration of seminal arginine decreases slightly, depending on the volumes of ejaculate [77] and vaginal discharge [78]. Thus, it is possible that the amount of arginine in the seminal fluid is sufficient to provide the survival of gonococci and the colonization of the female vaginal–cervical biotope [79].

It is generally acknowledged that the composition of the vaginal microbiota is extremely diverse and can vary from a moderate microbial spectrum, characterized by predominance of a small number of lactobacillus species, to complex anaerobic communities that cause the development of bacterial vaginosis (BV) [80–83]. BV can have many negative effects on reproductive health, including an increased risk of sexually transmitted infections [84–86], HIV [87, 88], premature delivery [89], pelvic inflammation [90], and cervicitis [91]. With bacterial vaginosis, the content of putrescine and cadaverine in the vaginal secretions significantly increases, while the concentration of their precursors, amino acids, especially arginine and ornithine, decreases. Putrescine and cadaverine in this biotope are produced mainly by bacteria [74, 92, 93], as evidenced by the inhibition of their accumulation in the presence of metronidazole [94], which has a bactericidal effect.

It is interesting to note that various inflammatory diseases of male genital tract are accompanied by a decrease in the polyamine concentration in the ejaculate and a significant change in their ratio [95]. It is worth noting that even with pronounced bacteriospermia, the

content of "bacterial" polyamines does not reach the values comparable to those in the vaginal fluid. Probably, the reason for this is the effect of acidity on the metabolism of polyamines, since the inducible BPA synthesizing enzymes have low optimal pH values [19], which are typical of the vagina. Polyamines in this case can be by-products that are formed due to bacterial protection against acid stress [96, 97]. On the other hand, it was shown that volatility of short chain polyamines increases with rising pH [98, 99]. It can also be the cause of low detectable concentrations of free putrescine and cadaverine in sperm plasma.

ALTERATION IN THE ANTIBIOTIC SUSCEPTIBILITY OF BACTERIA IN THE PRESENCE OF BPA

There is evidence that polyamines contribute to the survival of microorganisms under antibiotic treatment. This is mainly shown on gram-negative bacteria, such as *E. coli, Salmonella enterica, Pseudomonas aeruginosa,* and others [69, 70, 100]. A decrease in their susceptibility to various groups of antibacterial agents (beta-lactams, aminoglycosides, fluoroquinolones) in the presence of BPA has been experimentally proven [69, 70, 101]. Since the fluids of the urogenital tract, especially in men, are rich in polyamines (spermine and spermidine), it is believed that these compounds could protect gonococci from antimicrobial agents. In the available literature, we did not find such data. Polyamines are shown to protect gonococci against cationic antimicrobial peptides (polymyxin B and LL-37), but there is no protective effect under ciprofloxacin, spectinomycin, and penicillin treatment [102]. The absence of the effect of polyamines on the *N. gonorrhoeae* susceptibility to antibiotics looks rather unexpected, since their protective action is associated with such universal mechanisms as deactivation of ROS, which are forming in the cells under antibiotic effect [70], reduction of antimicrobial transport due to a decrease of the porin channel permeability [45] and other mechanisms. No doubt, this issue requires further studying.

CONCLUSION

It is well known that genital GI proceeds differently in men and women. The "male" gonorrhea is characterized by manifested symptoms and pus-like discharge. On the contrary, in women, the disease, as a rule, is not accompanied by typical symptoms, and they often learn about GI after infecting their sexual partner. In this respect, the differences in the spectrum and content of BPA in the ejaculate and vaginal discharge seem to be interesting. It is supposed that the manifestation of the process in the first case is largely determined by the reactivity of eukaryotic cells, and not by the metabolic activity of the microbiota of the male reproductive system. At the same time, the course of "female" gonorrhea is primarily determined by the state of the microbiota of the cervical–vaginal biotope, which is indirectly marked by the origin of the dominating polyamines. We believe that the diagnostic, prognostic and differentiating value of the content and spectrum of BPA in the reproductive tract of men and women with genital gonococcal infection and other sexually transmitted infections requires clarification.

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Virtual reality technologies in complex medical rehabilitation of patients with cerebral palsy

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ABSTRACT

The review highlights the issue of applying virtual reality (VR) technologies in medical rehabilitation of patients with cerebral palsy (CP). This review generalizes the current evidence on the use of virtual reality in restoration of motor and coordination functions, as well as in correction of other diseases associated with motor disorders in patients with CP. The analysis of national and international research shows that at present it is impossible to speak unambiguously about the efficiency of VR in rehabilitation of patients with CP. This is explained by some methodological shortcomings of the analyzed works (small size of the studied samples, lack of control over the results in the long term). However, the use of VR technologies for improving various functions in patients with CP is a promising method of medical rehabilitation.

Key words: virtual reality, rehabilitation, cerebral palsy.

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Технологии виртуальной реальности в комплексной медицинской реабилитации пациентов с детским церебральным параличом

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РЕЗЮМЕ

Обзор посвящен вопросам применения технологий виртуальной реальности (ВР) в медицинской реабилитации пациентов с детским церебральным параличом (ДЦП). Обобщены современные данные касательно использования ВР в восстановлении двигательных, координаторных функций, а также коррекции других расстройств, сопутствующих двигательным нарушениям у пациентов с ДЦП. Анализ

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работ, представленных в отечественной и зарубежной литературе, показывает, что в настоящее время нельзя однозначно говорить об эффективности ВР в реабилитации пациентов с ДЦП. Это связано с рядом методологических недостатков проанализированных работ (небольшой размер изучаемой выборки, отсутствие контроля результатов в отдаленный период). Тем не менее использование технологий ВР с целью улучшения различных функций у пациентов с ДЦП является перспективным методом медицинской реабилитации.

Ключевые слова: виртуальная реальность, реабилитация, детский церебральный паралич.

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INTRODUCTION

Cerebral palsy (CP) refers to a group of stable disorders of movement and posture maintenance that lead to motor defects due to non-progressive damage and / or abnormalities of the developing brain in a fetus or a newborn child [1–3]. CP is an urgent problem of modern medicine, as it is the main neurological cause of childhood disability worldwide, which affects families, education, and social life of the child [4]. The prevalence of CP is 2–3.6 cases per 1,000 children [5]. Competent choice of methods and terms of rehabilitation allows for social adaptation of children with this pathology, improving the prognosis of their motor and mental development [2].

There are many methods of medical rehabilitation of patients with CP at the moment. Their effectiveness depends on the patient's rehabilitation potential and a set of procedures [6]. The following rehabilitation measures are currently used: orthosis; physical rehabilitation (massage, therapeutic gymnastics, hardware kinesiotherapy, robotic mechanotherapy), including rehabilitation based on the principle of biofeedback; surgical orthopedic interventions on the extremities; surgical correction of spinal deformities in children with CP; botulinum therapy [7]. The main goal in the rehabilitation of patients with CP is to help the child achieve the highest possible level of physical, cognitive, psychological, and social independence [2].

Nevertheless, it remains relevant to develop and implement new effective and safe methods of rehabilitation of patients with CP, aimed at restoring their motor activity. At the same time, it is extremely important to use treatment methods that will be not only effective, but also interesting for children, and will result in patient's involvement in the rehabilitation process, which should affect the treatment outcome [8]. Motivation and active participation of children in the rehabilitation process play a key role [9].

Currently, virtual reality (VR) technologies are becoming more popular. VR technologies can significantly improve the results of rehabilitation treatment [10, 11]. VR is a computer simulation of a real environment which is able to evoke a sense of presence through 3D images and animations. VR provides interaction with various objects in this environment [12, 13]. VR technologies were initially used in the 50s of the XX century, usually for entertainment purposes. In the mid-60s, the potential for using VR was recognized by researchers from a variety of fields, including medicine [14]. VR technologies have shown positive results in the treatment of acrophobia [15], itching [16], pain syndromes [17], post-traumatic stress disorder, depression, and insomnia [18]. A high potential of VR was found in the rehabilitation of patients with CP [19], children with autism spectrum disorder [20–22], and patients with Parkinson's disease [23], Alzheimer's disease [24], and multiple sclerosis [25]. High efficiency of VR in medical rehabilitation is explained by achievement of the immersion effect, which allows to distract the patient's attention from pain and discomfort and reduce anxiety or dissatisfaction with treatment [26]. A sense of immersion in VR is provided by virtual reality glasses, special helmets, projectors, gloves with sensors, as well as the VRML (Virtual Reality Modeling Language) encoding language for describing three-dimensional images. The patient's participation or immersion in VR can be implemented in the following ways: active, when the user con-

trols a virtual image (avatar) or a specific VR object and passive, when videos are viewed without active control [27]. Virtual reality allows a person to interact with various objects in real time, unlike other forms of visualization (video games, television) [28]. A user in an artificially created virtual environment can have an experience similar to events and actions in real life [29]. The patient can see, feel, manipulate, move objects, and manage events in virtual space. This creates a "presence effect" [30]. The greatest effectiveness of using VR from the standpoint of evidence-based medicine was revealed when restoring the function of walking [31] and manipulative function of the upper limb [32], which was achieved by creating a virtual environment as close to the real one as possible, as well as by creating motivation for active participation of the patient in rehabilitation activities [32, 33]. It is the active involvement of the patient in the training process in the virtual environment, during which you can realize and correct your mistakes when performing movements, that allows to achieve high results in teaching the patient motor skills [34, 35]. VR training helps to effectively hone movements due to three key elements necessary for improving motor functions: repetition of stimulation, sensory feedback, and patient motivation [26, 35].

Restoration of motor disorders using VR is associated with activation of brain plasticity mechanisms, including changes in the primary sensorimotor cortex and supplementary motor area [35, 36]. This knowledge allows to expand the range of nosologies in which significant results can be achieved using VR [37, 38].

USING VR TECHNOLOGIES IN NEUROREHABILITATION IN PATIENTS WITH CP

The use of virtual reality as an additional method of rehabilitation is one of the promising directions in the correction of motor and concomitant disorders in patients with CP [39]. The use of virtual reality in children with CP is very popular, since computer technologies are motivating for children and young people [40]. It is worth noting that insufficient motivation may prevent the patient from reaching their functional potential [9, 41]. Virtual reality allows to perform complex movements in a safe environment and provides an opportunity for active learning [2, 9]. Some studies point to the positive impact of virtual reality on the reorganization of brain structures, neuroplasticity, motor abilities of the patient, visual perception, social behavior, and personal qualities [2, 19, 42]. A positive feature of using virtual reality is that the actual daily activity of the patient is taken into account [43].

There are many different VR systems, including Virtual Rehab, Caren, Nirvana, Tyromotion, MiTii, and others. One of the most commonly used systems is VirtualRehab, which is a rehabilitation platform that uses Microsoft Kinect and Leap Motion sensors, as well as video game technology. The VirtualRehab system is designed to restore the motor functions of the limbs. VR therapy using Microsoft Kinect and Nintendo Wii sensors has proven to be effective in improving exercise performance and increasing physical activity [44].

VR equipment is characterized by high cost and complexity, which determines the use of these systems only in the clinical setting. However, game systems are being developed for home use as well: TyromotionPablo for hand and finger training; Tymo for balance and strength training; the Interactive Rehabilitation Exercise System (IREX) with immersion and gesture control technology; YouGrabber and YouKicker systems for training upper and lower limb movements. The game form of treatment increases child's attention to performing certain exercises in comparison with conventional treatment [45, 46] and, most importantly, makes it possible to use VR technologies at home [47]. Currently, VR games for patients with CP are being actively developed and implemented in rehabilitation practice. These games will be therapeutically relevant and at the same time interesting for patients themselves [48]. In many studies on the use of VR in rehabilitation of children with CP, the effectiveness of the method is evaluated depending on the established goal: restoring the function of the upper and/or lower extremity, postural control and balance; improving physical fitness, and training the cardiovascular system [42, 49].

USING VR TECHNOLOGIES TO RESTORE UPPER LIMB FUNCTIONS IN PATIENTS WITH CP

One of the top priority tasks of restoring household and social activity of patients with CP is to improve the basic motor skills of the upper limb, such as the ability to coordinate movements of two hands, reach an object, and manipulate it [50].

J.W. Yoo et al. analyzed in their study the effectiveness of VR-based biofeedback using elec-

tromyography (EMG-biofeedback) in patients (n = 18) with various forms of CP (average age 9.5 ± 1.9 years) to improve the upper limb functions. All patients with cerebral palsy had one 30-minute EMG biofeedback session, followed one week later by another 30-minute VR-based EMG biofeedback session. The results were evaluated by the following tests: the range of motion of the elbow joint, the box and block test, and the biceps muscle strength test. Statistically significant improvement in all the tests was detected after the application of EMG biofeedback in conjunction with VR ($p < 0.05$). This study is the first clinical trial that demonstrated the effectiveness of using VR-based EMG biofeedback in patients with cerebral palsy to improve the upper limb functions [51].

G. Acar et al. studied the effectiveness of using VR (Nintendo Wii) to improve the upper limb functions in patients with hemiparetic form of CP (n = 30) with level I–II motor disorders according to the Global Motor Function Classification System (GMFCS). The patients were divided into two groups of 15 people depending on the complex of rehabilitation measures received. In the first group (average age 9.5 ± 3.0 years), traditional neurological treatment using VR was performed (30 minutes) (tennis, boxing, baseball – 5 minutes each); in the second group (average age 9.7 ± 2.9 years), only traditional neurological treatment was performed (45 minute-session). All patients received 45-minute treatment 2 times a week for 6 weeks. The upper limb function was evaluated before and after treatment using the Quality of Upper Extremity Skills Test (QUEST), the Jebsen – Taylor Hand Function Test, the ABILHAND test, and the Pediatric Functional Independence Measure (self-care). The results of the study demonstrated a more pronounced improvement in the upper limb function in patients with hemiplegic CP who were treated with traditional neurological treatment in conjunction with VR [52].

In addition, pilot studies were conducted that determined high efficiency of using VR systems to improve upper limb functions in patients with hemiplegic CP [47, 53].

One of the most popular systems for restoring upper limb functions and planning movements is the MiTii program ("move it to improve it"), which uses an interactive computer game that is controlled by hand and body movements. The system has remote configuration and progress check, as well as an opportunity to add personalized features to a series of games. Currently, randomized clinical trials of the MiTii VR system are being conducted at the University of Queensland (Australia) in order to evaluate the effectiveness of improving the upper limb function and to better understand the central neurovascular mechanisms that cause changes in the upper limb function, movement planning, and executive function [54].

Despite the positive results of using VR to improve the upper limb function in patients with CP, the data are controversial. In a systematic review of S. Rathinam and colleagues, only 4 out of 6 studies showed improvement. In 2 other studies, no changes were observed after the inclusion of VR in rehabilitation measures [55].

Thus, at the moment, it is impossible to claim high efficiency of using VR to restore the upper limb function in the rehabilitation of patients with CP. It is extremely important to monitor long-term results, in particular, to evaluate the application of skills acquired in the process of VR-based rehabilitation in real life situations and actions.

USING VR TECHNOLOGIES TO RESTORE LOWER LIMB FUNCTIONS (WALKING) IN PATIENTS WITH CP

Restoring lower limb functions in patients with CP is the key and most difficult task of medical rehabilitation. The aim of studies related to correction of motor disorders of the lower extremities in cerebral palsy is usually to improve walking [9].

S. Gagliardi and colleagues conducted a pilot study to evaluate the effectiveness of immersive VR used to improve walking in patients with CP. 16 children with spastic tetraplegia were included in the study (average age 11 ± 2.4 years). The rehabilitation was aimed at restoring walking and endurance skills using the Gait Real-time Analysis Interactive Lab (GRAIL). 18 sessions of therapy were conducted for all patients within 4 weeks. The effectiveness of the GRAIL VR system was evaluated using functional and instrumental methods, including gait analysis and Gross Motor Function Measure 88 (GMFM-88). Improved walking behavior (left and right step length (p = 0.001 and 0.003, respectively); walking speed (p = 0.001), endurance (6-minute walk test (p = 0.026)), gross motor functions (GMFM-88 (p = 0.041)), and other kinematic and kinetic parameters were observed 4 weeks after the start of rehabilitation activities using VR [56].

A.T.C. Booth et al. conducted a systematic review and meta-analysis of studies (from 1980 to 2017) evaluating the effectiveness of using VR to improve gait in children with CP. The meta-analysis contained 41 studies, including 11 controlled randomized trials. It was determined that the use of VR for functional gait training in patients with CP leads to clinically significant positive outcomes. It was found that functional gait training has moderate positive effects on walking speed compared to standard physical therapy ($p = 0.04$). There is weaker evidence that functional gait training using VR can improve walking and motor functions. The authors argue that functional gait training using VR is a safe, feasible, and effective method for improving walking in children with CP. Besides, adding virtual reality and biofeedback to rehabilitation activities in patients with CP can increase patient motivation and improve treatment outcomes [57].

However, there are also conflicting data regarding the effectiveness of virtual reality technologies in improving walking in patients with CP. D. Levac et al. conducted a pilot non-randomized controlled trial that included two groups of patients with different forms of CP. In the first group ($n = 5$), patients with CP had one VR session per day for 5 days in the hospital setting. After that, active video games were used at home for 6 weeks. In the second group ($n = 6$), only active video games at home for 6 weeks were used. Walking was evaluated in all patients using the 6-minute walk test (6MWT) and the global motor function scale (GMFM-88).

No differences were found between the groups based on the results of the study. In the group that used active video games at home for 6 weeks, a statistically significant improvement according to GMFM-88 ($p = 0.042$) was observed. In the first group, where VR was used in the clinical setting and active video games were used at home, better 6MWT values were found ($p = 0.043$). Despite this, all 6MWT values returned to their original level after 2 months. Thus, the authors concluded that neither VR nor active video games improved motor functions in patients with CP [58].

However, the use of VR to restore the lower limb function remains relevant. Currently, protocols have already been developed for clinical studies of the effectiveness of VR technologies for restoring lower limb functions (walking) in patients with CP [59, 60]. It's worth noting that these protocols allow to evaluate long-term results of rehabilitation measures.

USING VR TECHNOLOGIES IN RESTORING POSTURAL CONTROL AND MAINTAINING BALANCE (BALANCE, COORDINATION) IN PATIENTS WITH CP

Postural control, movement coordination, and balance are the key factors that provide most functional skills, especially walking and maintaining body position in space. The main reason for impairment of postural control is increased co-activation of agonist and antagonist muscles, as well as a decrease in the regulation of postural muscle contraction in a specific situation [61]. In recent years, some VR trials have focused on evaluating improvements in postural control and movement coordination in patients with CP [62, 63].

The first study using VR to restore postural control is the work by J.E. Deutsch et al., which presents a clinical example with retro- and prospective observation. The study included a 13-year-old child with spastic diplegia who was treated using the Nintendo Wii gaming system for 11 sessions of 60–90 minutes over 4 weeks. According to the results of observation, there was an improvement in postural control, visual perception, and functional mobility [64].

The study by S. Gordon et al. used the Nintendo Wii system as a method of rehabilitation of children with dyskinetic CP. 6 patients aged 6 to 12 years were included in the study. Two rehabilitation sessions a week for 6 weeks were performed. All children had improved postural control and motor functions as a result of using the virtual reality system [8].

16 subjects were included in the study by D. Sharan et al. A study group included 8 patients with CP (average age 8.9 ± 3.2 years), and a control group contained 8 children without pathology (average age 10.4 ± 4.4 years). The Nintendo Wii Sports and Wii Fit VR systems were used for rehabilitation. Motor activity was evaluated using the Manual Ability Classification System (MACS) and the Pediatric Balance Score (PBS). The positive effect was detected according to the PBS. There were no differences in manual skills compared to the control group. Thus, researchers showed that the use of Nintendo Wii Sports and Wii Fit VR systems had a positive effect on the function of balance control in the CP patient [29].

There are also other studies in which the use of technologies demonstrated high efficiency in improving postural control and balance maintenance in children with CP [65, 66].

In 2018, D. Cano Porras et al. presented a systematic review of 97 articles, 68 of which were published in 2013 and later. This review concludes that VR has a positive effect in restoring balance and gait, and also has advantages in combination with traditional rehabilitation methods [67]. VR together with transcranial magnetic stimulation has a positive effect on maintaining balance [68].

Thus, the inclusion of VR technologies in rehabilitation activities has positive prospects for improving postural control and balance in patients with CP.

USING VR TECHNOLOGIES TO IMPROVE PHYSICAL DEVELOPMENT AND CARDIOVASCULAR TRAINING IN PATIENTS WITH CP

Active video games are an optimal alternative to passive computer games and have recently become very popular among children and teenagers. Physical activity and physical fitness are reduced in children with CP compared to their healthy peers. Patients with CP spend most of their time sitting, also in front of a monitor screen [69].

Several studies evaluated the impact of active VR games on physical activity of children with CP when used at home. These studies showed that active games moderately improve physical activity, as well as reduce the time spent sitting in front of the monitor screen [70, 71].

INFLUENCE OF VR TECHNOLOGIES ON CONCOMITANT CP SYNDROMES

Most children with CP have comorbidities in addition to motor disorders. These disorders include behavioral, cognitive, and learning disabilities that affect general motor functions. 40% of patients with CP have attention deficit hyperactivity disorder [2]. Some studies exhibited a positive effect of VR-based methods on cognitive functions and behavioral disorders of patients with CP [72, 73].

M. Pourazar et al. presented the results of a randomized controlled trial on evaluating the effectiveness of VR in order to improve reaction time in children with CP. 30 boys aged 7–12 years were included in the study and divided into 2 groups (a study group and a control group). Measurement of reaction time (SRT, simple reaction time) and evaluation of discriminative reaction time (DRT) were performed in all patients initially and after day 1. A VR session between two dimensions was performed in the study group using the Xbox console. According to the results of the study, the reaction time decreased in patients with CP after using VR. The authors believe that VR systems are a promising tool in the rehabilitation process for improving reaction time in children with CP [74].

The possibilities of using VR to correct oral and facial disorders in children with CP are being studied. M. L. Martín-Ruiz et al. believe that performing rehabilitation activities using the VR-based SONRIE method on facial muscles can improve swallowing, facial muscle function, and speech in children with CP. All future studies will focus on SONRIE validation for correction of functional insufficiency of maxillofacial muscles in children with CP [75].

J.W. Shin et al. studied the effect of traditional neurological treatment and VR training programs on eye and hand coordination in children with CP. The study included 16 patients with diplegic CP. In the control group (n = 8), patients performed physical therapy exercises 2 times a week for 45 minutes for 8 weeks. In the study group (n = 8), patients performed physical therapy exercises (30 minutes) and VR training (15 minutes) 2 times a week. The results of the study showed a significant improvement in the coordination of eye and upper extremity movements in the study group. The authors claim that a properly planned training program using VR can improve eye and upper limb coordination in children with CP [76].

Thus, the use of VR technologies can improve limb function, walking, postural control, and balance in patients with CP. In addition, virtual reality technologies have a positive impact on such socially important functions of patients with CP as behavior, facial expressions, reaction time, hand-eye coordination, etc.

THE DISADVANTAGES OF USING VR TECHNOLOGIES

Despite the positive results of research on assessing the effectiveness of virtual reality in the rehabilitation of patients with CP, there are disadvantages associated with the use of VR. Until now, many VR systems have not been adapted for patients with CP with severe spasticity (the degree of spasticity is 2–4 according to the Ashworth scale). Here, games that require the use of a remote control are implied. Currently, games that can individually adapt to the reduced functions of a patient with CP are being developed [77]. For example, Sony has created a touch glove for the Sony PlayStation 3 game console, as well as several VR games for patients with cerebral palsy with

upper limb dysfunction [78]. There is also an Interactive Rehabilitation and Exercise System (IREX) that uses motion detection and capture technology, which facilitates the interaction of the patient with the game system [79].

In addition, most of the available virtual reality games may be too complex for patients with cerebral palsy. Special programs require the purchase of additional technical devices, and also have a high cost [9].

It is worth noting that virtual reality technologies contain a limited number of games. This reduces the motivation for long-term training. A study by S.G. Owners et al. noted a reduction in the time spent playing the game after 6 weeks of use. Besides, the duration of training using the Nintendo Wii Fit decreased by 82% in the first 6 weeks of use [80].

In addition, there are factors that limit the use of VR technologies. D. Levac et al. conducted a survey among Canadian physical and occupational therapists on the clinical use of VR in rehabilitation activities, as well as on the factors that prevent the use of VR. A total of 1,071 respondents were surveyed. Factors impeding the use of VR were a lack of funds, premises with the necessary space, time, staff, and patients with the necessary pathology [81].

It should be noted that the disadvantage of many works related to the use of VR technologies in the rehabilitation of children with CP is the small number of patients included in the study. This is explained by a number of limitations, such as the ethical aspect of using VR and parents' distrusting new rehabilitation methods [46, 81].

CONCLUSION

Currently, the inclusion of VR in the complex of rehabilitation measures for patients with CP is being studied. VR technologies create a three-dimensional virtual environment and are able to provide visual, audio, and tactile feedback for complete patient immersion. Thus, VR opens up new opportunities in the medical rehabilitation of patients with CP. The virtual environment provides optimal conditions for improving motor functions, postural control, balance, general motor activity, and associated syndromes. Interactive games increase motivation for therapy.

The potential role of virtual motor rehabilitation is promising. However, more information is required about its effectiveness and safety. At the moment, there are conflicting data regarding the use of VR technologies in the rehabilitation of patients with CP. This may be related to the size of the samples being studied, the timing of observation, and the estimated outcome indicators. Further development of VR technologies is necessary along with a detailed study of the effectiveness and safety of this rehabilitation method and its impact on the daily functional activity of patients with CP.

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Electrospinning for the design of medical supplies

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ABSTRACT

In this review, various achievements in the field of development of tissue-engineered scaffolds with the electrospinning approach were observed. Through the appropriate selection of electrospinning parameters, such as solution viscosity, the type of solvent, voltage, the distance between a tip and a collector etc., scaffolds with a high degree of porosity and pore size applicable for optimal cell infiltration can be obtained. These tissue-like materials can be produced from both synthetic and natural polymers and their mixtures. Based on the characteristics specific for the desirable tissue – vascular, bone or cardiac – materials providing the required mechanical properties, architecture, degradation kinetics and biocompatibility are selected for scaffold synthesis. In different studies, electrospun fibers were modified by adding biologically active agents or nanoparticles. This article also describes the particularities of the extracellular matrix of different tissues and approaches used for specific tissue imitation. Repopulation of the matrices with autologous cells before transplantation is the most commonly used method to improve the biocompatibility of the scaffold and the recipient.

Key words: tissue engineering, nanofibers, electrospinning, scaffolds, extracellular matrix, implants.

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Электроспиннинг для дизайна материалов медицинского назначения

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РЕЗЮМЕ

Рассмотрены достижения в области получения скаффолдов для тканевой инженерии методом электроспиннинга. При правильном подборе параметров электроспиннинга, таких как вязкость раствора, тип растворителя, напряжение, расстояние от иглы до коллектора и т.д., можно получить материалы с высокой степенью пористости и необходимым размером пор, подходящим для оптимальной инфильтрации клеток. Данные тканеподобные материалы можно получать как из синтетических и природных полимеров, так и их смесей. Исходя из свойств, присущих конкретной ткани – сосудистой, костной, сердечной и т.д., подбираются материалы для синтеза скаффолда, обеспечивающие необходимые механические характеристики, структуру, скорость деградации и биосовместимость. Многие исследователи функционализировали волокна путем добавления биологически активных веществ или наночастиц. В обзоре также рассмотрены особенности

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внеклеточного матрикса различных видов тканей и подходы, которые применяются для имитации ткани в каждом конкретном случае. Заселение скаффолдов клетками перед трансплантацией является наиболее распространенным подходом для повышения биосовместимости скаффолда с тканями реципиента.

Ключевые слова: тканевая инженерия, нановолокна, электроспиннинг, скаффолды, внеклеточный матрикс, имплантаты.

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

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INTRODUCTION

It is known that some organs and tissues of the human body are able to self-regenerate. For conditions when self-regeneration is limited, tissue-engineering approaches have been developed to create artificial tissues for reparation or organ replacement [1–3]. The electrospinning method is a new direction in this field, which makes it possible to create an implantable scaffold (prosthesis) with the required physicochemical characteristics [4]. The resulting porous structure facilitates active migration of cells into the wall of the prosthesis, formation both the supplying blood system and the internal endothelial layer. The electrospun scaffold should have a structure similar to the extracellular matrix of the replaced tissue to provide conditions for cell adhesion, growth and proliferation [5, 6]. Along with this, the implantable prosthesis should also have biocompatibility, a suitable pore size for cell infiltration and mechanical integrity [7, 8]. As a rule, such prostheses are made from biodegradable materials, which makes them a temporary supporting structure for the period of cell growth and tissue regeneration. The rate of degradation of the scaffold should coincide with the rate of new tissue formation.

ELECTROSPINNING PROCESS. EQUIPMENT AND MATERIALS

The basic elements of the electrospinning setup are a needle, through which a polymer solution is administered, and a collector, designed to carry the incoming polymer. These elements are combined into one electrical circuit. As the voltage increases, the surface tension forces of the polymer solution are overcome at the end of the needle, resulting in the formation of a Taylor cone – a tapered drop of polymer [9, 10]. As the voltage becomes sufficient, a polymer jet rises from the top of the cone in the direction of the collector, the diameter of which depends on many conditions. In the air, a part of the solvent evaporates and the jet splits, as a result of which a pure polymer is deposited on the collector in the form of randomly or directionally laid fibers with sizes in the nano- to micrometer range. The obtained material has the form of a thin, fibrous, porous soft fabric or thin elastic coating.

Structurally, the electrospinning setups are similar, the differences are associated only with the design (horizontal, vertical, etc.). In addition, setups with double electrospinning can be equipped with elements that control the formation of threads and fabric materials [11]. An interesting technology is the coaxial electrospinning, which allows to obtain combined (like a braided wire) threads [12, 13].

The electrospinning process is influenced both by the properties of the solution (viscosity, electrical conductivity, polymer concentration, surface tension) and controlled variables: the rate of the solution feed or polymer dissolution, the amplitude of the electric voltage, the distance between the needle and the collector, temperature, and humidity [14, 15].

The viscosity, electrical conductivity and surface tension of polymer solutions depend on the concentration and properties of the polymer and solvent used. Typically, the viscosity of solutions in electrospinning processes is in the range of 1–20 poise (Ps) and depends on the concentration (usually in the range of 10–30%) and the molecular weight of the polymer [16, 17]. Optimum solvents should have low viscosity and a low boiling point (for example, DCM, THF, DMF, water, methanol, hexafluoroisopropanol, etc.); solvent mixtures are also used [18–20]. The surface tension of the solutions is also determined by the nature of the solvent and the polymer (usually about 10^2–10^3 dyne / cm), although in practice this factor is rarely controlled.

The main controlled parameters, such as the polymer feed rate (from 0.1 to 10 ml/h), the voltage value (from 1 kV to 60 kV), the distance between the needle and the collector (from 10 cm to 50 cm), the diameter of the nee-

dle (18–27G), and the speed of rotation of the receiving collector (0–3000 rpm), are determined experimentally. In the vast majority of cases, the temperature and humidity in an electrospinning installation are not considered.

Electrospinning technology is suitable for manufacturing polymer filaments, tangles and films from soluble or molten polymers and allows to create fibrous materials with specified spatial characteristics (diameter, spatial orientation and adhesion of fibers, porosity, the presence of channels for cell proliferation). Thanks to this, materials for cell and tissue engineering are created and studied: various scaffolds for nerve tissue, skin, bone tissue, etc.; dressings; drug delivery vehicles [21–23].

BIODEGRADABLE VASCULAR GRAFT PRODUCTION

The ability to combine the advantages of synthetic and natural polymers via electrospinning makes this method particularly attractive for the design of vascular grafts requiring high mechanical tensile strength and sufficient elasticity (Young's modulus) [24]. In addition, the inclusion of natural polymers with a large number of cell binding sites in the scaffold can contribute to the formation of a continuous monolayer of epithelial cells in the lumen and the proliferation of other types of cells in the graft matrix. The electrospinning method provides precise control of the composition, size and direction of the fibers, which affects the porosity of the material, pore size distribution, and scaffold architecture [25]. It is important to note that directed nanofibers can be used to orient cells in a certain direction to provide the necessary anisotropy that occurs in some organs, including blood vessels [26].

A group of authors [27] obtained tubular scaffolds from a copolymer of poly-L-lactide and poly-ε-caprolactone with a diameter of 3 mm, which were implanted into rabbits in the lower superficial epigastric veins for a period of 7 weeks. It was revealed that the frames withstood the surgical process, retained structural integrity and patency throughout the observation period. In addition, endothelial cells obtained from the human coronary artery were evenly distributed and spread well throughout the carcass cavity within 10 days after application.

Vascular grafts obtained from a solution of recombinant human tropoelastin and polycaprolactone were chosen in [28] to simulate the mechanical properties of the human thoracic artery (elastic modulus, ductility, permeability, and rupture pressure). Cell adhesion and proliferation were investigated using human umbilical vein endothelial cells. The cell-free framework was implanted into rabbits and removed one month later, followed by a study of the mechanical characteristics. In the case of transplants of elastin / polycaprolactone composition, increased vascular compatibility and endothelization were observed with reduced platelet attachment compared with transplants without elastin. The addition of tropoelastin significantly improved cell adhesion and proliferation.

Polyurethane–urea grafts were implanted in rat aorta for up to 24 weeks [29]. The interior of the grafts was coated with a non-thrombogenic 2-methacryloyloxyethyl phosphorylcholine copolymer, which resulted in reduced platelet adhesion and improved patency compared to uncoated grafts. The mechanical properties of the grafts were also compatible with those of native arteries. Numerous *in vivo* experiments using composite bilayer polyurethane–urea grafts with applied muscle stem cells of rats, introduced into their aorta [30, 31], showed higher throughput for grafts with applied cells compared to grafts without cells.

Vascular grafts from biodegradable polyurethane were implanted in rat abdominal aorta for the periods of 7 days, 1 month, 6 months, and 12 months [32]. A comparison was made with commercially available ePTFE grafts. In all cases, rejection of the implants by the body or their degradation was not observed. With a long implantation period, the implant patency was 100%. The implant removed after 12 months remained mechanically stable and was fully integrated into the surrounding tissue.

The emulsion electrospinning method was used to make heparin-filled poly (L-lactide-co-caprolactone) nanofibers (PLCL) used as a stent coating. In a rabbit model, it was found that a stent coated in such a way effectively separated the aneurysm from the bloodstream [33]. In another work, Wang et al. [34] mixed vascular endothelial growth factor (VEGF) with heparin to accelerate endothelialization and loaded the resulting mixture into the core of PLCL nanofibers. Isolation of heparin and VEGF from PLCL-Hep-VEGF scaffolds lasted more than 30 days, which increased the proliferation of porcine iliac endothelial cells on the stent.

Feng et al. obtained coxial electrospinning stents coated with PLCL nanofibers filled with heparin and calcium rosuvastatin [35]. The coated stent showed increased anticoagulant ability, and the endothelial cells proliferated well on the coated stent due to the prolonged release of rosuvastatin calcium and heparin (more than 45 days) from PLCL coaxial nanofibers.

In a similar work by Chen et al., heparin and VEGF were encapsulated in PLCL nanofibers by emulsion electrospinning to create vascular grafts [36]. Heparin and VEGF showed a sustained release for 29 days, which gave the studied transplant good anticoagulation ability and led to the growth of endothelium.

Similarly, platelet-rich growth factor (PRGF) at a

concentration of 20 mg / ml was added to the PLCL to prepare an electrospinning solution from which a tubular graft with a fiber diameter of 4 nm was prepared. This approach facilitated rapid penetration of cells into the graft and the growth of endothelium [37].

A group of authors [38] investigated the release of tritium-labeled paclitaxel (3H-PTX) from matrices designed to coat vascular stents and obtained by electrospinning from solutions of polycaprolactone (PCL) with paclitaxel (PTX) and human serum albumin (HSA) in hexafluoroisoropropanol (HFIP). It was shown that 3D matrices can completely release PTX with virtually no weight loss. Approximately 27% of PTX was released on the first day, another 8% – in the next 26 days. Given the toxicity of PTX and the rate of diffusion through the arterial wall, it is expected that the minimum cytostatic dose of the drug in the artery wall will be maintained for at least three months.

MATERIALS FOR SKIN REGENERATION AND WOUND HEALING

High porosity, small pore size and large surface area of electrospinning coating materials make them suitable for wound dressings, where they provide effective protection against bacterial infection of the damaged skin surface and the ability to drain wound fluids and gases through the dressing. Electrospinning coatings can also work as platforms for the delivery of biologically active substances, such as antimicrobial agents to fight infections and agents to improve wound healing [39, 40].

The main component of the extracellular matrix of the skin is collagen fibers that provide mechanical and structural integrity of the skin and have diameters in the range of 50–500 nm [41, 42]. Therefore, any material for skin regeneration should also have a fiber diameter in the nanometer range. Among natural polymers, collagen, fibroin, gelatin, and chitosan / chitin are often used for this purpose. Thus, a group of authors [43] obtained collagen nanofibers with a diameter of 100–1200 nm by electrospinning. The mechanical properties of the collagen matrix were comparable to those for commercially available materials for regeneration of damaged tissues. In experiments on rats, the matrices obtained were highly effective in the treatment of wounds at early stages.

Fibroin secreted from silkworm cocoons is characterized by excellent biocompatibility, high strength, slow degradation, and minimal inflammatory response [44]. However, the material formed during electrospinning has a small pore size, which prevents proper cell infiltration. A group of researchers obtained fibers from a mixture of silk fibroin and polyethylene oxide with the simultaneous deposition of NaCl crystals during electrospinning [45]. Good adhesion and infiltration of 3T3 fibroblasts on the matrix were observed. In rat wound treatment experiments, this scaffold closed wounds faster and degraded more efficiently than the commercially available MatriDerm® regenerative material.

Synthetic polymers (polyglycolide, polylactide, poloxamer, polycaprolactone, polystyrene, polyvinylpyrrolidone, etc.) are also used for skin regeneration. A group of authors [46] conducted a comparative study of fibers obtained from polycaprolactone, a mixture of chitosan / polyethylene oxide and gelatin by electrospinning their solutions in acetic acid. In *in vitro* tests, the material of the chitosan / polyethylene oxide composition was characterized by low cell adhesion and proliferation, while during the *in vivo* study in rats it had the greatest influence on the treatment process, effectively blocking wound contraction and enhancing its epithelization. The polycaprolactone scaffold also performed poorly in *in vitro* experiments, but acted well as a physical barrier against wound contraction. When using gelatin, the best *in vitro* results were observed, while the *in vivo* behavior was comparable to the control group (wound regeneration without the use of a scaffold).

The method of electrospinning of a polyurethane solution produced nanofibers with an average diameter of 250–300 nm [47]. A study on the treatment of wounds using a polyurethane membrane was carried out on guinea pigs; the control group received treatment with the commercial product Tegaderm ™. Good, uniform adhesion of the nanofiber membrane to the wound surface was observed without accumulation of fluid. At the same time, its toxic effects or permeability to exogenous microorganisms were not revealed.

El-Aassar et al. [48] developed electrospinning composite wound coatings containing polyvinyl alcohol (PVA) / Pluronic F127 (Plur) / polyethyleneimine (PEI) and titanium dioxide nanoparticles (TiO2 NP). In this study, TiO2 nanoparticles were used as an antimicrobial agent. Antibacterial tests showed that the fabricated PVA-Plur-PEI / TiO2 nanofibers exhibited better bactericidal activity than PVA-Plur-PEI nanofibers.

Lv et al. [49] reported a polycaprolactone (PCL)/ gelatin electrospinning framework containing silica-based bioceramic particles (Nagelschmidtite, NAGEL, Ca7P2Si2O16) for wound healing. Using the joint electrospinning process, NAGEL bioceramic particles were uniformly distributed in PCL / gelatin fibers, and when the framework was destroyed, silicon-containing ions (silicates) were gradually released from the fiber. Cell tests (for example, umbilical vein endothelial cells (HUVECs) and human keratinocytes (HaCaTs)) showed that scaffolds can significantly contribute to cell adhesion, proliferation and migration. The wound sites reconstructed by these scaffolds showed the desired healing

results in terms of angiogenesis, collagen deposition, re-epithelialization, and inhibition of the inflammatory response.

MATERIALS FOR BONE TISSUE, TENDON AND LIGAMENT REPARATION

The key points when using scaffolds obtained by electrospinning for bone tissue regeneration are the presence of a system of interconnected pores, proper mechanical properties, a controlled degradation rate, and fiber size corresponding to the structure of the extracellular matrix of the bone [50]. The extracellular matrix of the bone is a nanocomposite consisting of collagen fibers, inorganic nanocrystallites, and growth factors [51].

The possibility of obtaining scaffolds for bone tissue regeneration from polycaprolactone was studied [52]. The polycaprolactone nanofiber matrix obtained by electrospinning from a chloroform solution consisted of randomly oriented fibers with diameters ranging from 100 nm to 5 μm.

Scaffolds coated with rat bone marrow mesenchymal stem cells were cultured with osteogenic additives in the bioreactor for 4 weeks with the following implantation in the omentum. After removal, the scaffolds retained their original size and shape, were stiff and looked like a bone. Throughout the matrix, the formation of cells and extracellular matrix was observed with the formation of tissue similar to that of the bone.

Hydroxyapatite (HA), as the main mineral component of the bone matrix, is widely used in medicine for bone tissue reconstruction [53], but its use is limited by its inherent high fragility. One of the solutions to this problem is to obtain composite materials with polymers [54–56]. Thus, a nanofiber network of collagen and hydroxyapatite (HA content of about 20%) was obtained by electrospinning a solution of their nanocomposite [57]. The biocompatibility of nanofibers was investigated using mouse osteoblastic cells. The cells were viable and showed good growth parameters both in the case of collagen nanofibers and in the case of nanocomposite.

Scaffolds made from silk fibroin fibers containing human recombinant bone morphogenetic protein (BMP-2) and / or hydroxyapatite nanoparticles obtained by electrospinning were used to form bone tissue *in vitro* from human mesenchymal bone marrow stem cells [58]. BMP-2 underwent the process of electrospinning in the aquatic environment and retained bioactivity. Cells were cultured on scaffolds for 31 days in an osteogenic environment. The scaffold supported the processes of growth and osteogenic differentiation of cells. Co-application of BMP-2 and hydroxyapatite onto the fibers resulted in the highest levels of calcium deposition and increased the levels of BMP-2 transcription compared to other systems. However, BMP-2 has disadvantages associated with rapid enzymatic hydrolysis, undesired bone growth, immune responses, and high cost [59]. Recently, peptides derived from BMP-2 [60], which have a positive effect on osteogenic differentiation of stem cells and bone formation in defects [61], have attracted great attention as alternative biologically active molecules.

Ye et al. [62] developed nanoscale 3D frameworks of nano-hydroxyapatite / PLLA / gelatin (nHA / PLA / GEL) with immobilized on them derivatives of BMP-2 peptides, capable of delayed release. *In vitro* studies have shown that nHA / PLA / GEL-PEP scaffolds stimulate bone mesenchymal stem cell alkaline phosphatase activity (BMSCs) and gene expression associated with osteogenic differentiation. In addition, in an *in vivo* model, this scaffold promoted bone formation in defects of rat cranial bones. Hence, it is stated that this framework has great potential for repairing bone defects [62].

Native ligaments and tendons have a wide range of mechanical properties. Therefore, for the successful creation of tissues replacing these damaged structures, the mechanical properties of the scaffold play a crucial role [63]. Hybrid nano-microfiber scaffolds were developed by electrospinning a solution of polylactide-co-glycolide onto an already prepared scaffold woven from fibers of the same polymer [64]. Pig bone marrow stromal cells were applied to hybrid scaffolds, and woven scaffolds without nanofibers, onto which cells suspended in a fibrin gel were applied, were used as a control group. Indicators, such as cell adhesion, proliferation, and expression of type I collagen and decorin, were higher for the hybrid scaffold group. A limitation in the use of such nano-microfiber scaffolds is their mechanical properties, which do not correspond to those characteristic of native tendons and ligaments.

MATERIALS FOR CARDIAC TISSUE REGENERATION

Tissue engineering and cell therapy are now seen as alternative therapeutic approaches to stimulate the regeneration of infarcted tissue. Cardiac scaffolds can replace or support the function of the heart muscle with the possibility of providing cell and drug therapy after myocardial infarction [65, 66]. Both synthetic and natural polymers are used to produce heart scaffolds.

In [67], the production of matrices of different composition by electrospinning was described: poly-L-lactide; 75% polylactide-co-glycolide (lactide / glycolide = 10 / 90) mixed with 25% poly-L-lactide; 85% polylactide-co-glycolide (lactide / glycolide = 75 / 25) mixed with 15% copolymer of polyethylene glycol with poly-D, L-lactide. During the cultivation of cardiomy-

ocytes on the obtained matrices, it was revealed that cardiomyocytes were sensitive to the composition of materials with a preference for relatively hydrophobic surfaces. The density of cardiomyocytes on hydrophilic and rapidly decomposing surfaces was lower. Matrices of poly-L-lactide showed the best parameters for cell adhesion.

The work [68] describes the preparation of composite fibers, the core of which consists of polyglycerol sebacate, and the outer part – of fibrinogen. The fibers had an average diameter of 1076 ± 212 nm. It was shown that the obtained fibers had a Young's modulus comparable to that of the native heart muscle. Neonatal cardiomyocytes that were cultured on these scaffolds showed normal expression of specific proteins.

A number of scaffolds with directed and randomly oriented fibers were obtained by electrospinning from a mixture of polyglycerol sebacate / gelatin with different ratios of components [69]. The adhesion, proliferation, and differentiation of fibroblasts and cardiomyocytes were influenced by the chemical composition and stiffness of the scaffold, and the alignment and organization of cells was influenced by the directed or random arrangement of the fibers. Scaffolds with directional fibers containing 33% polyglycerol sebacate allowed to achieve optimal synchronous contractions of cardiomyocytes with significantly improved cell organization in given directions.

Some conductive and biocompatible polymers, such as polyaniline and polypyrrole, have been used to make conductive cardiac scaffolds. In particular, a scaffold was made from directional conductive fibers of polyaniline and a copolymer of polylactic and polyglycolic acids [70]. Cells cultured on fibers formed clusters, and all cardiomyocytes within the cluster contracted synchronously, which implies a fully developed intercellular connection.

Another approach to improve the physical and biological properties of a scaffold is to coat its surface with nanofibers. In some cases, scaffolds coated with electrospinning nanofibers, after removing cells from the surface of the scaffold, showed better mechanical properties and kinetics of degradation than before modification [71]. For example, a group of researchers conducted an experiment to obtain hybrid leaflets of the heart valve with a biomatrix / polymer composition [72]. The leaflets of the pig's heart valve were purified from cells and coated with a mixture of fibroblast / chitosan / poly-4-hydroxybutyrate growth factors by electrospinning. Further, the leaflets were seeded with rat mesenchymal stem cells and cultured for 14 days. As a result, the hybrid scaffold showed good cell population and a significant increase in their mass, the formation of 4-hydroxyproline and collagen, and also had mechanical properties comparable to those of the native valve.

In a recent work [73], a fiber frame of a polycarbonate–polyurethane valve was developed by electrospinning, which imitates the shape of a native heart valve. According to the authors, the cell-free, slowly collapsing elastomeric valve implant should be gradually populated by endogenous cells with the formation of new valve tissue inside the heart. Orthotopic implants in the form of a pulmonary valve in sheep showed stable functionality for up to 12 months, while the polymer implant was gradually replaced by layered collagen and an elastic matrix as the cells resorbed.

As for medical bioresistant implants, for materials obtained by electrospinning, the scope has not yet been determined. For implants, especially artificial heart valves (AHV), increased operational requirements are imposed: long-term elasticity, wear resistance, biostability, not to mention hemocompatibility and resistance to calcification. To date, biological AHV with moving elements from an animal pericardium demonstrate the greatest success in practice, although in this case it is also difficult to achieve long trouble-free lifetime of the valve (usually no more than 10 years) [74]. Artificial biomimetic heart valves with a movable base made of artificial materials (polymers, such as PTFE, polyurethanes, etc.) are currently considered promising, but need more in-depth study than the already known mechanical or biological ones [74]. Therefore, the technology of electrospinning should be rather considered as an auxiliary method in the production of combined biostable materials. For example, electrospinning microfibers can be used as a reinforcing component when creating the appropriate composite (valve prosthesis, vessel, graft) with an impregnated polymer matrix or to modify the surface layer of the product with nanofibrils for better epithelization where necessary.

CONCLUSION

The task of tissue engineering is to restore the functions of damaged tissue. The electrospinning method allows to obtain polymer scaffolds for the needs in this area. By varying the parameters of electrospinning, it is possible to obtain materials with the required fiber diameter, pore size and porosity. In addition, electrospinning allows to obtain fibers from various polymers, both of synthetic and natural origin. This technology allows to combine the advantages of synthetic and natural polymers to obtain biocompatible scaffolds with mechanical properties corresponding to native tissues. The selection of conditions and materials for electrospinning is carried out depending on the properties of the extracellular matrix of the replaced tissue. However, today, the number

of *in vivo* studies for tissue-engineering materials obtained by electrospinning is still insufficient to talk about the operational flexibility of this technology.

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Authors contribution

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Antimicrobial resistance monitoring: a review of information resources

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ABSTRACT

In the last decades, the problem of antibiotic resistance has been occupying one of the key positions in the global public health system, and it requires attention from the medical community. Antimicrobial resistance monitoring systems play an important role in tracking the changes in antimicrobial susceptibility for timely correction of antimicrobial therapy. The pharmaceutical industry applies epidemiological data obtained through such monitoring to the creation of new medicines and modification of the antimicrobial substances developed earlier.

The article describes some of the international and Russian monitoring systems created at different times. It should be noted that during development, regional-level data are used, while a number of projects present information on a global scale. The completed comparative analysis of available systems revealed both positive aspects and parameters in need of renovation. At the same time, the standardization of collecting basic data for monitoring programs requires significant changes. The majority of systems are able to examine only a limited range of microorganisms and antimicrobials. An important point in the functioning of monitoring systems is a search for the optimal way to visualize output data in tables, interactive maps, and graphics. A significant amount of projects demand further work on the result presentation options. Constant monitoring is a significant component in modern concepts of antibiotic resistance control due to the increasing occurrence of resistant organisms.

Key words: analytical systems, antibiotic resistance, antimicrobials, surveillance.

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Мониторинг антибиотикорезистентности: обзор информационных ресурсов

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РЕЗЮМЕ

В последние десятилетия проблема антибиотикорезистентности занимает одну из ключевых позиций в системах общественного здравоохранения во всем мире и требует особого внимания со стороны медицинского сообщества. Для отслеживания динамики чувствительности микроорганизмов, с целью

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своевременной коррекции антимикробной терапии, важную роль играют системы мониторинга антибиотикорезистентности. Полученные в рамках мониторинга эпидемиологические данные также используются в фармацевтической промышленности при создании новых лекарственных препаратов и модификации разработанных ранее антимикробных субстанций. В статье рассматриваются некоторые из зарубежных и российских систем мониторинга, созданные в разное время. Следует отметить, что при разработке используются данные на уровне отдельных регионов, а ряд проектов представляют информацию в глобальном масштабе. Результаты проведенного сравнительного анализа имеющихся систем выявили как положительные стороны, так и параметры, которые требуют модернизации. При этом в наибольших изменениях нуждается процесс стандартизации сбора исходных данных для программ мониторинга. Большинство систем лимитировано по спектру рассматриваемых микроорганизмов и антимикробных препаратов. Важным моментом для функционирования систем мониторинга является поиск оптимального способа визуализации выходных данных при помощи таблиц, интерактивных карт и графиков. Значительное количество проектов требует дальнейшей проработки вариантов представления результатов. Возрастающая частота резистентных микроорганизмов требует постоянного мониторинга, являющегося важной составляющей современных концепций сдерживания антибиотикорезистентности.

Ключевые слова: системы анализа, антибиотикорезистентность, антимикробные препараты, эпидемиологический надзор.

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

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INTRODUCTION

For the first time, reports on the emergence of antimicrobial resistance appeared in the 1940s. The first cases registered were connected with penicillin resistance. Then, as various antimicrobials were introduced into clinical practice, virtually all known pathogens acquired resistance to one or more drugs [1]. As a consequence, antibiotic resistance monitoring is a key element in the fight against this threat. Its main components include continuous collection, analysis, and interpretation of information related to antibiotic resistance. A monitoring system with the ability to permanently track the dynamics of antibiotic resistance in all clinically significant isolates, studied in healthcare facilities, can be considered impeccable. The data on resistance rates are suitable for research programs on infection control and the rational use of antibiotics. They are also used in the development of practical measures aimed at reducing antibiotic resistance [2].

Antibiotic resistance monitoring programs are able to indicate regions where the problem is widespread, geographical objects with increased rates of resistance, and microbial species that pose a serious threat to public health [3–5]. Moreover, the monitoring system is an early warning tool, and its efficiency depends on the time needed for interested stakeholders to access the information and their ability to take actions. At the same time, access to up-to-date and reliable information on the antibiotic resistance level contributes to the formation of an adequate response to private reports of antibiotic resistance in cases involving an improper assessment of the expected drug efficacy, which may consequently complicate antimicrobial therapy [2, 6].

Several key objectives are pursued in developing an antibiotic resistance monitoring system: to detect, analyze and predict changes in antibiotic resistance indicators and outbreaks of infections caused by microorganisms with modified resistance; to detect new resistance mechanisms; to compare the activity of new antibiotics before and after their introduction into clinical practice. In addition, a key point is the opportunity to educate medical staff, patients, and general public on antibiotic resistance issues.

It should be noted that for about 30 years since the detection of resistant microorganisms, the concept of monitoring antibiotic resistance has not existed in its modern sense. Only individual publications describing cases of antimicrobial ineffectiveness existed.

INTERNATIONAL ANTIBIOTIC RESISTANCE MONITORING PROGRAMS

Indirectly, antibiotic resistance monitoring systems have been in place since 1970, when several US clinics reported cases of hospital-acquired infections [7]. The immediate task was to collect data on nosocomial infections and susceptibility of germs to antimicrobials. This local system subsequently became the basis of the USA National Nosocomial Infection Surveillance System [8, 9].

Modernization of antibiotic resistance monitoring programs unequivocally shows that the quality of the organization has reached a new level. At present, the following levels of current initiatives to monitor antibiotic resistance can be determined: local, regional, national, and international. In addition, the levels can be hierarchical and different sources of funding can be applied to the systems [10, 11]. For instance, in a number of European countries, microbiological laboratories working on antibiotic resistance issues are obliged to provide the results of the observations to concerned organizations and healthcare providers at least once a year [12]. Moreover, local monitoring is realized by hospital laboratories, systematically reporting on the microbial susceptibilities. Europe, the USA and other countries develop regional, national, and international surveillance systems [8, 13, 14].

The group of national initiatives for antibiotic resistance monitoring includes Active Bacterial Core Surveillance (ABC system, USA). It was created as part of an infection research program in collaboration with the Centers for Disease Control and Prevention (CDC, USA) to assess the severity of invasive bacterial infections, which in a significant number of cases are manifested as sepsis and meningitis [4]. This project website published reports on the incidence of infections caused by different groups of *Streptococcus*, *H. influenzae*, MRSA (Methicillin Resistant *Staphylococcus Aureus*), *N. meningitidis* and contains information on some demographic characteristics [15]. The system also comprises a trend estimation of infectious diseases in the territory of several American states employing both molecular and microbiological methods.

Furthermore, the following programs exist: National Nosocomial Infections Surveillance (NNIS system, USA) and Global Emerging Infections Surveillance (GEIS, USA) [16, 17]. It should be noted that the NNIS report combines information from 300 clinics for the period from 1992 to 2004 and contains data on the frequency of nosocomial infections and various demographic indicators [18]. Limited information on antibiotic resistance, which includes only particular pathogens, is one of the main disadvantages.

In the early 2000s, several systems were combined into a single National Healthcare Safety Network (NHSN, USA). The final data of this program for 2006–2008 provide information about infections caused by different medical interventions and the use of diagnostic and therapeutic devices [19]. NHSN established an online monitoring system for antibiotic resistance in catheter-associated urinary tract infections, central line-associated bloodstream infections, and surgical site infections, in order to provide timely and complete data [20]. The structural elements of the application are an introduction page containing information on data collection methods; summary indicators of antibiotic resistance; and a map and an interactive table on selected microorganisms. Moreover, a graphic display of susceptibility indicators according to the patient's age, type of surgical interventions and health facilities is available to the user in the additional section. There are some drawbacks of the system, the main ones being a limited spectrum of antimicrobials and microorganisms and a lack of choice in localization of infections and hospital units. The database adopted as the basis for NHSH is not updated regularly; the latest available information dates back to 2015.

Public Health England (PHE) provides direct financial support in the development of several antibiotic resistance monitoring projects – Second generation surveillance system (SGSS) and the British Society for Antimicrobial Chemotherapy (BSAC). The characteristic feature of SGSS is presentation of summary information in the form of a report on the frequency characteristics of selected microorganisms and their susceptibility to antibiotics. The disadvantage of this system is the absence of possibility to access the website as an unregistered user. Moreover, the system contains only generalized data and non-interactive visualization [10]. BSAC involves participation of several countries: England, Wales, Scotland, Northern Ireland, and the Republic of Ireland [6]. The system forms publications and provides information with selection filters via the website; the output data cover the strain distribution of all susceptibility categories and minimum inhibitory

concentration (MIC) distribution, as well as genetic markers of antibiotic resistance. Weak points include the lack of graphical and cartographic representation of data and the inability to consider data at primary aggregation levels.

The Centers for Disease Control and Prevention (CDC, USA) conducted a study on antibiotic resistance in 10 countries within reinforcement of resources for surveillance, response and emerging infectious disease control. The implementation of activities depended on the country. A research project in Egypt included a university and several public hospitals to monitor nosocomial infections and antibiotic resistance. The demographic analysis was the basis for systematic surveillance in Thailand and several African countries, such as Kenya [21].

International projects on antibiotic resistance monitoring include the European Antimicrobial Resistance Surveillance Network (EARS-Net) [13], the Latin American Surveillance Network of Antimicrobial Resistance (ReLAVRA – acronym in Spanish) [22], the Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAESAR) [23], the Asian Network for Surveillance of Resistant Pathogens (ANSORP) [24], the Antibiotic Resistance Surveillance and Control in the Mediterranean Region (ARMed) [25,26], the Gonococcal Antimicrobial Surveillance Programme (GASP) [27], international research "Bacterial Infections and Antibiotic Resistant Diseases among Young Children in Low-Income Countries" (BIRDY) [28, 29], CDDEP(the Center for Disease Dynamics, Economics & Policy)-ResistanceMap [30], and the Global Antimicrobial Resistance Surveillance System (GLASS) [31].

EARS-Net was organized with financial support from the European Centre for Disease Control (ECDC) in 1999. EARS-Net 2018 report contained data on antimicrobial resistance of *E. coli, K. pneumoniae, P. aeruginosa, S. pneumoniae, Acinetobacter* spp., *S. aureus, E. faecalis, E. faecium* with selected antimicrobials being tested; and blood and cerebrospinal fluid were clinical materials [13, 32]. The data from the previous year are the base for an annual report. In addition, the trend in the dynamics of antimicrobial resistance for the last three years is shown in the form of tables and maps with gradient colors. The monitoring results are presented as an interactive system, not only in PDF. The main negative characteristics of the system are the limited spectrum of microorganisms and antibiotics and delayed access to results.

The Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAESAR) includes European countries that are not part of EARS-Net and several Central Asia countries. The organization is financially supported by the WHO Regional Office for Europe (the World Health Organization Regional Office for Europe); the Dutch National Institute for Public Health and the Environment, the Ministry of Health, Welfare and Sport (the Netherlands); and the European Society of Clinical Microbiology and Infectious Diseases. At this time, 19 countries are members of CAESAR. The first CAESAR report was published in 2014 and included data from 9 member countries that submitted results of antimicrobial susceptibility testing (AST) for 8 species (*E. coli, P. aeruginosa, K. pneumoniae, Acinetobacter* spp*., S. pneumoniae, S. aureus, E. faecalis* and *E. faecium*), with blood and cerebrospinal fluid as clinical materials. The report structure consists of introductory information about the project, participating countries, and the specific data collection. The outcome of AST is presented in the form of tables for each country [23]. The system is insufficient in the selection of comparative indicators. There are no options to choose the localization of infections and hospital units, and data presentation abilities are limited. In addition, the final data contain a certain number of microorganisms with only delayed access to the monitoring results.

WHO and the Pan American Health Organization collectively founded the Latin American Surveillance Network of Antimicrobial Resistance (ReLAVRA). The network includes 19 countries that share their data on antibiotic resistance annually. Individual publications and the official web portal present the results of the system. Limited possibilities of data visualization and user-configurable filtering and the absence of in-depth data analysis can be considered as disadvantages of the system [22].

Professor Jung-Han (the Samsung medical center, Sungkyunkwan University, Seoul, South Korea) initiated the Asian Network for Surveillance of Resistant Pathogens (ANSORP). The main goal of the project is to implement international prospective studies on antibiotic resistance in the Asian region. The initial project developed by the ANSORP group addressed the pneumococcal resistance monitoring. The resource is updated at certain time intervals and

generates reports on the results of prospective studies. In 2018, a working group of researchers began to work on collecting isolates and processing data in pediatric patients [24]. However, since the data presentation is limited to reporting, the expected difficulties in timely data presentation for the user and incomplete coverage of microorganism species arise. The visual component is also one of the underdeveloped aspects of the project.

Over a fouryear period, the Antibiotic Resistance Surveillance and Control in the Mediterranean Region (ARMed) functioned under the sponsorship of the Directorate General for Education and Culture. Cyprus, Egypt, Jordan, Malta (coordinator), Morocco, Tunisia, Algeria, Lebanon, and Turkey participated in the program. Susceptibility rates of *S. aureus, S. pneumoniae, E. coli, E. faecium,* and *E. faecalis* invasive strains and correctness of antibiotics prescription and intake were assessed. Due to the termination of external funding, ARMed has suspended its work [25].

CDDEP ResistanceMap is a website developed with support from the Center for Disease Dynamics, Economics and Policy (Washington, DC; USA) non-governmental organization. The resource presents data on consumption of antibacterial drugs and antibiotic resistance for different regions of the world and individual states [30]. The website provides information on 12 species of microorganisms. Visual presentation of data is possible in several variants: an interactive map, a trend and a bar chart. The system combines the results from several sources, such as the Australian Group on Antimicrobial Resistance (AGAR), the European Antimicrobial Resistance Surveillance Network (EARS-Net), the Canadian Antimicrobial Resistance Alliance (CARA), a private tertiarycare hospital (Kenya), the Public Health Surveillance, Institute of Environmental Science and Research Ltd (ESR, New Zealand), etc. [30]. Despite the big amount of information selected to form the ResistanceMap database, the potential system effectiveness is reduced by the scope of microbial selection, presentation options, and delayed data publication due to the dependence on primary sources.

One of the key positions of the global action plan on antibiotic resistance adopted at the World Health Assembly in 2015 is to strengthen the evidence base in this area [31]. The main ways are to conduct research and monitoring continuously on a global scale. This plan includes the development of the Global Antimicrobial Resistance Surveillance System (GLASS). A road map for five-year work was formulated for this project. During this period, antibiotic resistance is monitored based on clinical and microbiological data on priority bacterial pathogens, with the target of 40% WHO countries participating by the end of the project [33, 34].

Along with national and international general medical organizations, a number of pharmaceutical companies provide material support for the creation and maintenance of antibiotic resistance monitoring systems. Alexander Project, MYSTIC (Meropenem Yearly Susceptibility Test Information Collection), SENTRY, and TRUST are examples of such systems [35–39].

The SMART (Study for Monitoring Antimicrobial Resistance Trends) is also one of such projects, sponsored by the Merck & Co., Inc. The key objective of this system is to assess *in vitro* susceptibility of Gram-negative microorganisms to 12 antibiotics for intraabdominal and urinary tract infections. This website is designed for professionals working outside the USA [11]. The work results are available as an interactive map, publications and posters starting from 2009. Restricted choice of antibiotics and microorganism species and summary information display without detailed explanation limit the range of SMART applications.

ATLAS, a system supported by Pfizer, is a multi-component resource that includes a database that combines information from three sources: TEST (Tigecycline Evaluation Surveillance Trial), AWARE (Assessing Worldwide Antimicrobial Resistance Evaluation), and INFORM (International Network for Optimal Resistance Monitoring). The system currently obtains data on over 630,000 isolates. The user has a choice of parameters to filter the data by microorganism species, antimicrobials, geographic regions, and years. The output information is available as a heat map, a trend, or a table [40]. Point evaluation of indicators (city category) and multiple comparisons are not available, which emphasizes the need for further development of the project.

DEVELOPMENT AND IMPLEMENTATION OF ANTIBIOTIC RESISTANCE MONITORING SYSTEMS IN THE RUSSIAN FEDERATION

National antimicrobial resistance monitoring projects are regularly organized in the Russian Federation with the direct participation of the Institute

of Antimicrobial Chemotherapy (IAC) and the Interregional Association for Clinical Microbiology and Antimicrobial Chemotherapy (IACMAC); they are managed by the Scientific-Methodical Center on Antibiotic Resistance Monitoring (CMAR) [41]. Since the 1990s, there have been various projects, such as RESORT, REVANSH, MARATHON, Pegas, START, NOTA, MARS, STENT, SPARS, Darmis, and NPRS [42]. The results of the projects were presented in electronic reports with tables and graphs. The publication time was delayed for several months, and only statistical data without user-configurable settings were presented.

AMRmap, an online platform for the analysis of antimicrobial resistance data in Russia, has been in function since 2016. Its significant difference is a wide range of tools for visualization, with a choice of a data format: charts, tables or interactive maps. The graphical module varies from bar charts to more rare variants – matrices and graphs. It is necessary to note the implemented system of filters, which allows to receive results on the scale of both federal districts and cities. Methods for assessment of associated resistance and presentation of genetic determinants of antibiotic resistance were designed for AMRmap [43]. The database, which the website is based on, includes the results of multicenter prospective antibiotic resistance studies conducted by IAC and IACMAC covering the period from 1997 to present, with retesting of obtained isolates at IAC central laboratory. Despite continuous updates, the process of input data generating requires further development, with an improvement of data implementation in the platform.

CONCLUSION

Constant high quality work of monitoring systems is an important element in controlling antibiotic resistance. The greatest efficiency in practical application is possible in the conditions of constant database filling, and expansion of criteria forming it. The best option is to conduct monitoring based on multicenter studies with the participation of all healthcare facility types in the project. At the moment, most of the local data come from large hospitals, which reflect the resistance rates due to a larger number of patients. Thus, regular inclusion of new participants in the antibiotic resistance monitoring, with an undiminishing quality and intensity of research protocol processing, will help to assess the situation with antimicrobial resistance more accurately. The data obtained will be used to improve monitoring systems, and the information provided will timely optimize antibacterial therapy on a global and national scale.

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Neurogenic inflammation: biochemical markers, genetic control and diseases

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ABSTRACT

Neurogenic inflammation is a pathological process based on bidirectional interactions between cells of the nervous and immune systems as well as on a wide range of biologically active substances.

Aim. Basing on scientific publications and information provided in databases, to analyze markers of neurogenic inflammation (biochemical, genetic) and characterize their involvement in the pathogenesis of diseases of various organ systems.

Results. Neurogenic inflammation that occurs during the development of various diseases (asthma, urticaria, atopic dermatitis, psoriasis, rheumatoid arthritis, pain syndrome, interstitial cystitis, colitis, etc.) is characterized by common stages and pathophysiologically active substances. Mediators released by nerve cells (substance P, calcitonin gene-related peptide, vasoactive peptide), acting on specific receptors, contribute to mast cell degranulation with the release of a complex of biologically active substances (histamine, tryptase, nerve growth factor, etc.), which activate inflammatory processes. Biologically active substances and receptors significant for the development of neurogenic inflammation are under genetic control. At the same time, there are overlaps of the spectrum of diseases for which importance in the pathogenesis of neurogenic inflammation is proved and an association between variants of neurogenic inflammation genes. This makes it possible to conclude that the course of neurogenic inflammation will depend not only on the etiological factors, but also on the genetic features of key molecules involved in neurogenic inflammation processes. The similarity of the pathogenetic links of neurogenic inflammation (at the genetic and biochemical levels) in various pathologies may underlie the formation of comorbid conditions.

Conclusions. Understanding the biochemical and genetic components of the development of neurogenic inflammation is of interest for prevention and treatment of diseases (including comorbid ones) based on this pathological process.

Key words: neurogenic inflammation, genetics.

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Нейрогенное воспаление: биохимические маркеры, генетический контроль и болезни

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РЕЗЮМЕ

Актуальность. Нейрогенное воспаление представляет собой патологический процесс, в основе которого находятся двунаправленные взаимодействия между клетками нервной и иммунной систем, а также широкий спектр биологически активных веществ.

Цель. На основании научных публикаций и информации, представленной в базах данных, проанализировать маркеры нейрогенного воспаления (биохимические, генетические) и охарактеризовать их вовлеченность в патогенез болезней различных систем органов.

Результаты. Нейрогенное воспаление, протекающее при развитии различных заболеваний (астма, крапивница, атопический дерматит, псориаз, ревматоидный артрит, болевой синдром, интерстициальный цистит, колит и др.), характеризуется общностью этапов и патофизиологически активных веществ. Выделяемые нервными клетками медиаторы (субстанция Р, кокальцигенин, вазоактивный пептид), воздействуя на специфические рецепторы, способствуют дегрануляции тучных клеток с высвобождением комплекса биологически активных веществ (гистамин, триптаза, ростовой фактор нервов и др.), которые активируют воспалительный процесс. Биологически активные вещества и рецепторы, значимые для развития нейрогенного воспаления, находятся под генетическим контролем.

При этом наблюдается перекрывания спектра заболеваний, для которых доказана значимость в патогенезе нейрогенного воспаления, с одной стороны, и ассоциированность с вариантами генов нейрогенного воспаления – с другой. Это позволяет заключить, что характер течения нейрогенного воспаления будет зависеть не только от этиологических факторов, но и от генетических особенностей ключевых молекул, вовлеченных в процессы нейрогенного воспаления. Общность патогенетических звеньев нейрогенного воспаления (на генетическом и патогенетическом уровнях) при различных патологиях может лежать в основе формирования коморбидных состояний.

Заключение. Понимание биохимических и генетических компонент развития нейрогенного воспаления представляет интерес для профилактики и лечения заболеваний (в том числе и коморбидных), в основе которых лежит данный патологический процесс.

Ключевые слова: нейрогенное воспаление, генетика.

Ключевые слова: системы анализа, антибиотикорезистентность, антимикробные препараты, эпидемиологический надзор.

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INTRODUCTION

The relationship between the nervous and immune systems is actively studied in terms of their involvement in the pathogenesis of various diseases, and in this aspect, neurogenic inflammation is of particular interest [1, 2]. Neurogenic inflammation is understood as a cascade of pathogenetically significant events due to the local release of inflammatory mediators from nerve cells in response to non-infectious stimuli [3–5]. The effect of certain stimuli (allergens, chemicals, etc.) on the nerve endings triggers processes involving different types of immune cells, peripheral and central nervous system nerve fibers, and numerous physiologically active substances. In particular, it has

been proven that, with the development of allergic inflammation, neurons actively interact and regulate the functioning of mast cells, dendritic cells, eosinophils, Th2 cells, etc., and complex and often bidirectional relationships are formed between different types of cells [6, 7]. The pathogenesis of a large number of diseases and/or symptoms of diseases (including asthma, urticaria, contact dermatitis, atopic dermatitis, psoriasis, rheumatoid arthritis, pain syndrome, autism, cystitis, etc.) is associated with neurogenic inflammation, and their spectrum is constantly increasing [1, 2, 8–18]. The bronchopulmonary system, gastrointestinal tract, urogenital system and skin are the most well-studied systems in terms of processes occurring in the development of neurogenic inflammation, as they are primarily exposed to various damaging exogenous agents. Clinical and experimental data accumulated so far show similarities between the stages of neurogenic inflammation in different organ systems [6, 11, 19, 20].

BIOCHEMICAL MARKERS OF NEUROGENIC INFLAMMATION

In a generalized (and simplified) form, the development of the neurogenic inflammatory process can be presented in the following way (Fig. 1). In response to irritation, the sensory neurons located in the epithelial layer of the skin, respiratory tract, gastrointestinal tract, and urinary system secrete neuropeptides (substance P – SP, vasoactive intestinal peptide – VIP, calcitonin gene-related peptide – CGRP), which activate mast cells by acting on appropriate receptors (NK1, MRGPRX2, VIPR1, CGRP-R).

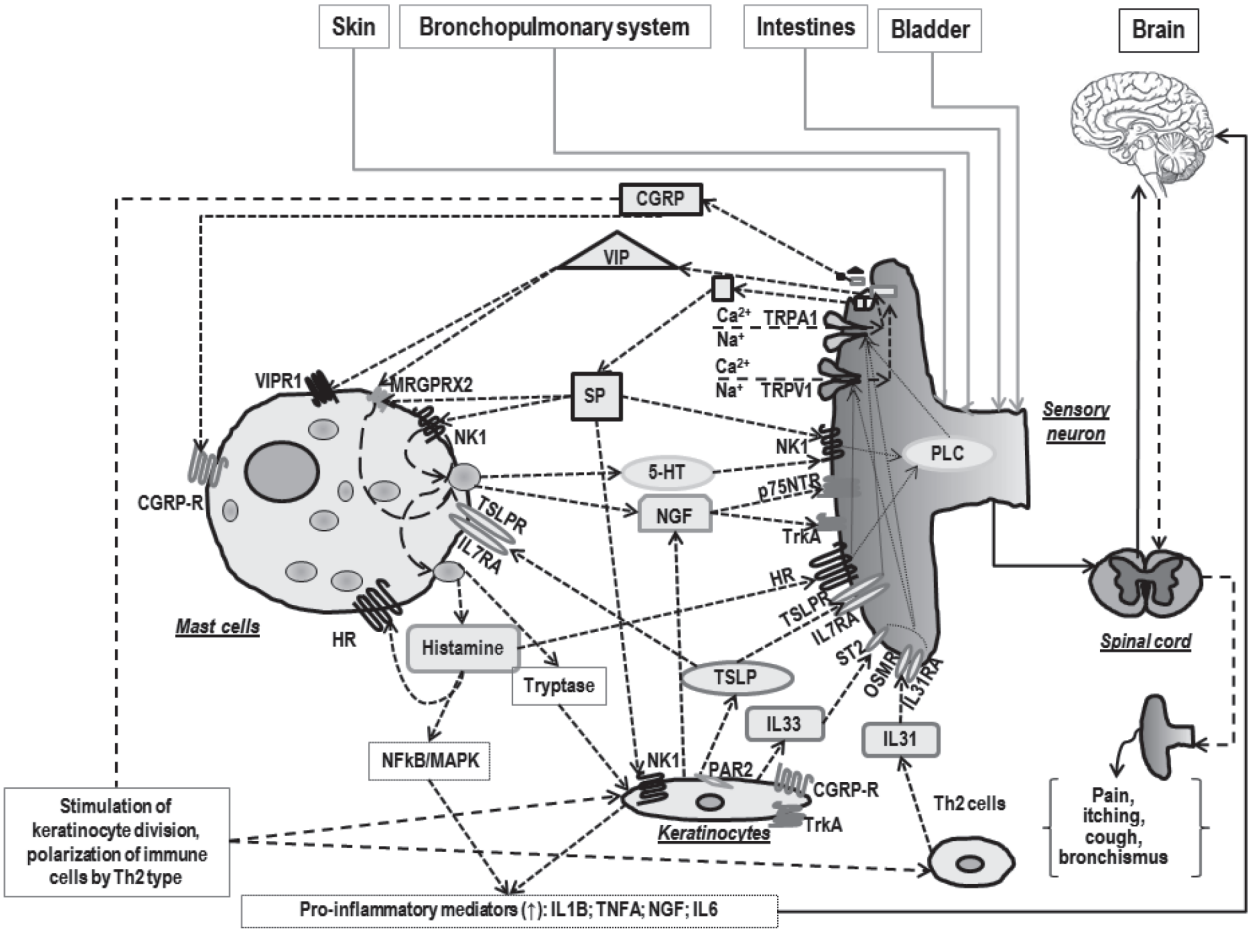

Figure 1. Schematic representation of the development of neurogenic inflammation (compiled from [6, 21, 22]). SP is substance P, NK1 is substance P receptor; VIP – vasoactive intestinal peptide, VIPR1 and MRGPRX – vasoactive intestinal polypeptide receptors; CGRP – calcitonin gene-related peptide, CGRP-R – receptor for calcitonin gene-related peptide; 5-HT – serotonin, NGF – nerve growth factor, p75NTR and TrkA – NGF receptors; TRPV1 and TRPA1 – calcium channels; IL1B, IL6, IL31, IL33 – interleukin-1B, -6, -31, -33, respectively, TNFα – tumor necrosis factor alpha, TSLP – thymic stromal lymphopoietin; OSMR and IL17RA form the receptor for TSLP and IL31; ST2 – receptor for IL33; HR – histamine receptors (four types of histamine receptors are known – H1R, H2R, H3R, and H4R), PLC – phospholipase C; MAPK – mitogen-activated protein kinase, NFkB – nuclear factor kappa-b. Details – see text

Following the activation, various physiologically active substances are released from mast cells – histamine, tryptase, serotonin (5-HT), nerve growth factor (NGF), and others. Histamine, by activating the NFkB/MAPK metabolic pathway, promotes the production of pro-inflammatory cytokines (IL1B, TNFA, NGF, IL6), resulting in the development of the inflammatory process. Influencing the receptors localized on nervous fibers, histamine, IL31 produced by Th2 cells, IL33, NGF, and TSLP (thymic stromal lymphopoietin) produced by tryptase-activated keratinocytes (in the case of a pathological process affecting the skin, as in allergies, for example) are capable of activating TRPV1 and TRPA1 calcium channels. The activation of calcium channels increases the entry of calcium ions into cells, which, in turn, promotes the release of neuropeptides, thereby increasing the immuno-inflammatory response. As a result, pathological reactions, such as itching, pain, bronchospasm, cough, sneezing, bowel spasm, abdominal pain, etc., are observed, i.e. there is a wide range of pathological reactions, depending on which organ develops neuroinflammation. In addition, CGRP released by skin sensory neurons in response to irritation not only induces polarization of immune cells along the Th2 pathway, but also stimulates keratinocyte division [6].

It is worth noting that the same biologically active substances (histamine, NGF, TSLP) are able to activate different types of cells and are involved in various pathophysiological processes, and some of them (histamine and substance P) have both paracrine and autocrine mechanisms of regulation, which complicates the development of the pathological process. In general, mediators involved in neuroinflammation, such as substance P, histamine, and 5-HT form a self-regulating cycle around the calcium channels, and regardless of the stage at which it started, even minor activation of more than one stage results in synergistic activation of the entire cycle [21]. This indicates that potentially neurogenic inflammation can act as a significant component of the pathogenesis for a wide range of diseases, regardless of the etiological factor, if there is an imbalance in the synthesis/degradation of neuroinflammatory mediators. For example, pathological processes by the type of neurogenic inflammation are recorded both in case of allergic asthma [1] and in case of exposure to tobacco smoke (in tissues of lungs, nasal mucosa, larynx, and brain) [4, 23].

Similar (or very close) mechanisms of inflammatory process development are observed in other pathologies. The development of neurodegenerative processes (Alzheimer's disease, Parkinson's disease, multiple sclerosis) involves inflammatory and neurotoxic mediators, such as IL1B, IL33, TNFα, substance P, and others [20], which are released by mast cells, neurons, and other cell types. This results in the increased concentration of Ca ions within cells and activation of mitogen activated kinase-like protein (MAPK) and nuclear factor NF-kappa-B (NFkB). In addition, when the blood-brain barrier is damaged (due to the progression of neurodegenerative diseases or for some other reason), immune cells and inflammation mediators may enter the brain from the periphery, which enhances neuroinflammation and neurodegenerative processes in these diseases.

According to I.V. Stagineva and A.G. Volkov [24], the division of facial pain symptoms in diseases of perinasal sinuses into somatic and neurogenic ones is justified only in terms of etiological factors, while in clinical manifestation of diseases, the pathogenetic mechanisms are inseparable. Involvement of substance P, histamine, and other substances significant for development of neurogenic inflammation in pathophysiological processes in rheumatoid arthritis [11], psychogenic urticaria [8], rhinosinusitis [13], facial pain in rhinosinusitis [24], cerebral dysfunction in chronic tobacco smoking [23], psoriasis [10], and cystitis [18] was established. Interestingly, histamine, being one of the key molecules of neurogenic inflammation, can not only provoke the development of well-known allergic pathological reactions of the skin (itching, urticaria) and bronchopulmonary system (nasal congestion, rhinorrhea, bronchospasm, etc.), but also lead to disruption of the cardiovascular and nervous systems (arrhythmias, anaphylaxis, hypotension/hypertension, dizziness, headache, migraine, vomiting, excitement, body temperature regulation, etc.), gastrointestinal tract (flatulence, abdominal pain), genitourinary system (dysmenorrhea, interstitial cystitis) , etc. [3, 25, 26]. Considering the importance of this amine for the development of neurogenic inflammation, it can be assumed that the neurogenic component may be significant to a certain extent for all these pathologies.

Neurogenic inflammation is a protective reaction of the body in response to certain exogenous stimuli. However, under certain conditions (long-term stimulus exposure, imbalance in the regulation of substances stimulating and inhibiting neurogenic inflammation, etc.), this pathophysiological process may become chronic.

ASSOCIATIONS BETWEEN GENES WHICH PRODUCTS ARE INVOLVED IN NEUROGENIC INFLAMMATION AND DISEASES

The development of neurogenic inflammation involves numerous molecules of different functional classes, the structural and functional properties of which depend on the genes encoding them or the genes which products are involved in the synthesis and degradation of such biologically active substances (in particular, histamine, serotonin synthesis). Neurogenic inflammation genes have been studied to varying degrees in terms of association with diseases and/or pathological phenotypes (table 1). Tumor necrosis factor alpha gene (*TNF*) and interleukin 1β (*IL1B*) gene were most frequently used for analysis, single reports are available for the tryptase beta 2 gene (*TPSB2*) and one MAS related GPR family member X2 (*MRGPRX2*). However, already at this stage of research, based on the accumulated data, it can be concluded that the overwhelming number of neurogenic inflammation genes can be characterized as highly pleiotropic (wherein the pleiotropy index is higher the more the gene studied). Yet, a high pleiotropy index of genes is combined with a low specificity index, i.e. gene variants more often show associations not with the disease, but with phenotypes (signs) and disease groups. It can be assumed that in this case the genes of neurogenic inflammation are more likely to act as genetic background determining the response to a particular exogenous stimulus (etiological factor) than as main causal factors of disease development.

Table 1

Functional "loading" of genes which products are involved in the processes of neurogenic inflammation*

Genes**	Total associated diseases/phenotypes	*DPI* – Disease Pleiotropy Index	*DSI* – Disease Specificity Index	The number of associated SNPs
TNF#	1640	0.966	0.263	21
IL1B	1035	0.931	0.312	15
PTGS2	832	0.897	0.338	24
POMC	557	0.862	0.382	20
NGF#	323	0.862	0.426	5
NTRK1	306	0.759	0.443	36
TRPV4#	289	0.759	0.519	51
CALCA#	274	0.828	0.456	1
TAC1#	252	0.793	0.473	–
TRPV1#	173	0.724	0.532	5
F2RL1	165	0.724	0.514	1
IL33	164	0.793	0.503	18
NGFR	160	0.724	0.508	2
VIP	152	0.759	0.526	2
DDC	131	0.621	0.554	50
IL7R	121	0.724	0.538	19
TSLP	107	0.690	0.547	9
TACR1#	105	0.724	0.559	6
MCAM	85	0.621	0.572	1
TRPA1	74	0.621	0.639	4
APP	71	0.862	0.430	63
PLCG1	62	0.517	0.611	4
VIPR1	56	0.483	0.624	2
IL1RL1	54	0.586	0.616	38
PNOC	52	0.414	0.672	–
HRH1	45	0.414	0.685	2
CRLF2	43	0.483	0.639	2
IL31RA	42	0.517	0.645	4
HDC	39	0.517	0.639	6
OSMR	38	0.448	0.648	8
HRH3	37	0.345	0.648	2

Table 1 (continued)

Genes**	Total associated diseases/phenotypes	*DPI* – Disease Pleiotropy Index	*DSI* – Disease Specificity Index	The number of associated SNPs
AMN	36	0.517	0.701	19
HRH2	32	0.310	0.663	–
HRH4	32	0.379	0.676	8
VIPR2	31	0.483	0.667	2
CIRBP	30	0.448	0.663	–
TPH2	28	0.552	0.562	28
IL31	23	0.276	0.727	1
CALCRL	21	0.517	0.707	2
MRGPRX1	21	0.483	0.752	–
TPSAB1	15	0.345	0.762	–
TPSD1	11	0.276	0.799	–
TPSG1	11	0.276	0.773	–
TPSB2	2	0.103	1.0	–
MRGPRX2	1	0.069	1.0	–

Note. * The information is taken from the DisGeNET database [27, 28]. ** Included genes are categorized as "neurogenic inflammation" according to DisGeNET (in bold) and according to scientific publications (neurogenic inflammation proteins shown in the diagram, Fig. 1). Gene *TNF* encodes TNFA protein, *NTRK1* gene – TrkA; *CALCA* gene – CGRP, *TAC1* gene – substance P (SP), *F2RL1* gene – PAR2, *NGFR* gene – p75NTR, *DDC* and *TPH2* gene products are involved in serotonin synthesis (5-HT), *IL7R* gene encodes IL7RA, gene *TACR1* – NK1 receptor, *PLCG1* gene – PLC protein, *IL1RL1* gene – ST2 receptor, *HRH1*, *HRH2*, *HRH3,* and *HRH4* genes – 4 types of histamine receptors (HR), respectively, *CRLF2* gene – TSLPR, *HDC* gene – an enzyme involved in the synthesis endogenous histamine; *CALCRL* gene – CGRP-R, genes *TPSAB1*, *TPSD1*, *TPSG,* and *TPSB2* genes encode tryptase; names of other genes correspond to the name of the proteins they encode. # Genes that are related to neurogenic inflammation according to various sources used for analysis were noted. *DPI* – Disease Specificity Index: the higher the index, the greater the number of different classes of diseases (MeSH) associated with the gene; Disease Specificity Index – *DSI* –varies from 0.25 to 1; the larger the index, the smaller the number of diseases associated with the gene.

For example, variants of the *TAC1* gene (encoding substance P) are associated with a wide range of diseases and phenotypes (depressive disorder, mental depression, gastroesophageal reflux disease, hyperreactivity of the bronchi, hyperemia, cirrhosis, colitis, pain, itching, tactile allodynia, inflammation, hyperalgesia (secondary), neuralgia, fibrosis, hypotension, bradycardia, allergic reactions, edema, etc.). Some of these diseases are also associated with variants of the *TACR1* gene encoding the substance P receptor (including hyperalgesia, mental depression, bradycardia, tactile allodynia, colitis, etc.) [27, 28]. This is consistent with the pathogenetic significance of proteins encoded by these genes in the universal neurogenic component of the pathogenesis of a wide range of diseases of various organs and systems.

As indirectly evidenced by associative studies, the spectrum of diseases for which neurogenic inflammation may be pathogenically significant is wider than that currently recognized. Neurogenic inflammation genes are overrepresented among genes associated with diseases of various organ systems (that is, they are recorded more frequently than if associations of the corresponding diseases with these genes were randomly detected) (table 2). Given the functional significance of neurogenic inflammation genes, it is expected that they are overrepresented among genes associated with such diseases/signs as hyperalgesia, swelling, inflammation and pain, itching, asthma, atopic dermatitis, and psycho–neurological disorders. In addition, genes of this pathological process are overrepresented among genes associated with diseases of the cardiovascular system (hypotension, hypertension, atherosclerosis, bradycardia, etc.), gastrointestinal tract (colitis), urogenital system (glomerulonephritis, albuminuria, cystitis), etc. For example, according to the DisGeNET database [27, 28], associations between the variants *DDC*, *HRH1*, *IL1B*, *PNOC*, *POMC*, *TAC1*, *TNF*, and *VIP* and hypotension were established (for 8 out of 82 genes associated with this pathology, the achieved level of the false discovery rate was FDR = $1.27 \cdot 10^{-6}$, see tab. 2); *CALCA*, *HRH3*, *IL1B*, *POMC*, *PTGS2*, *NACR1*, *TNF* were associated with hypertension (for 7 out of 302, FDR = 0.029), *HRH1*, *PTGS2*, *TNF*, *TRPV1*– with atherosclerosis (for 4 out of 59, FDR = 0.0164), *IL1B*, *NGF*, *POMC*, *TNF* – with glomerulonefrity (for 4 out of 40, FDR = 0.004), *IL1B*, *PTGS2*, *TNF* – with colitis (for 3 out of 40, FDR = 0.031), etc. In general, these associations are easily explained in view of the importance of

Table 2

Results of enrichment analysis for genes which products are involved in neurogenic inflammation

Disease/symptom	Calculated parameters*				
	N	*E*	*R*	*p*	*FDR*
Hyperalgesia	80	0.349	34.36	$4.44 \cdot 10^{-16}$	$1.14 \cdot 10^{-12}$
Edema	69	0.301	26.56	$4.25 \cdot 10^{-10}$	$7.25 \cdot 10^{-7}$
Bronchial hyperreactivity	12	0.052	95.44	$9.42 \cdot 10^{-10}$	$1.02 \cdot 10^{-6}$
Inflammation	114	0.498	18.08	$1.00 \cdot 10^{-9}$	$1.02 \cdot 10^{-6}$
Pain	79	0.344	23.20	$1.28 \cdot 10^{-9}$	$1.01 \cdot 10^{-6}$
Hypotension	82	0.358	22.35	$1.74 \cdot 10^{-9}$	$1.27 \cdot 10^{-6}$
Pruritus	59	0.258	27.18	$4.96 \cdot 10^{-9}$	$3.17 \cdot 10^{-6}$
Amnesia	17	0.074	67.378	$7.24 \cdot 10^{-9}$	$4.12 \cdot 10^{-6}$
Mental depression	260	1.135	9.69	$8.37 \cdot 10^{-8}$	$4.28 \cdot 10^{-6}$
Experimental arthritis	40	0.175	34.36	$1.60 \cdot 10^{-8}$	$7.42 \cdot 10^{-6}$
Bipolar disorder	516	2.253	5.77	$1.39 \cdot 10^{-7}$	$5.93 \cdot 10^{-5}$
Depressive disorder	413	1.803	6.100	$9.37 \cdot 10^{-7}$	$3.69 \cdot 10^{-4}$
Substance withdrawal syndrome	53	0.231	21.61	$3.00 \cdot 10^{-6}$	0.0011
Asthma	99	0.432	13.88	$3.86 \cdot 10^{-6}$	0.0013
Glomerulonephritis	34	0.149	26.95	$1.31 \cdot 10^{-5}$	0.0041
Mood disorders	187	0.816	8.57	$1.14 \cdot 10^{-5}$	0.0041
Fever	127	0.554	10.82	$1.63 \cdot 10^{-5}$	0.0044
Trigeminal neuralgia	12	0.052	57.26	$1.65 \cdot 10^{-5}$	0.0044
Atopic dermatitis	37	0.161	24.76	$1.85 \cdot 10^{-5}$	0.0047
Hyperemia	13	0.057	52.86	$2.14 \cdot 10^{-5}$	0.0052
Memory disorders	40	0.175	22.91	$2.53 \cdot 10^{-5}$	0.0059
Neuralgia	14	0.061	49.08	$2.71 \cdot 10^{-5}$	0.0060
Multiple sclerosis	42	0.183	21.81	$3.08 \cdot 10^{-5}$	0.0066
Gastric ulcer	18	0.0795	38.18	$6.00 \cdot 10^{-5}$	0.0123
Schizophrenia	1,041	4.54	3.08	$7.06 \cdot 10^{-5}$	0.0139
Necrosis	53	0.231	17.88	$7.79 \cdot 10^{-5}$	0.0148
Lung injury	20	0.087	34.36	$8.33 \cdot 10^{-5}$	0.015
Cerebrovascular accident	57	0.276	14.49	$1.53 \cdot 10^{-4}$	$9.46 \cdot 10^{-3}$
Overactive Bladder/Cystitis/Hereditary sensory and autonomic neuropathies/Acral ulceration and osteomyelitis leading to autoamputation/Anthracosis/HIV wasting syndrome**	4	0.017	114.53	$1.11 \cdot 10^{-4}$	0.016
Major depressive disorder	262	1.144	6.12	$1.19 \cdot 10^{-4}$	0.016
Atherosclerosis	59	0.258	15.53	$1.19 \cdot 10^{-4}$	0.016
Nerve degeneration	120	0.524	9.54	$1.61 \cdot 10^{-4}$	0.022
Albuminuria	25	0.109	27.49	$1.66 \cdot 10^{-4}$	0.022
Hereditary sensory autonomic neuropathy, type 5/Absence of pain sensation/Polymyositis**	5	0.022	91.62	$1.84 \cdot 10^{-4}$	0.022
Learning disorders	29	0.127	23.70	$2.60 \cdot 10^{-4}$	0.029
Congenital pain insensitivity/Sleep–wake disorders/Infection/ Postmenopausal osteoporosis /Dermatomyositis/Extravasation of diagnostic and therapeutic materials**	6	0.026	76.35	$2.76 \cdot 10^{-4}$	0.029
Hypertensive disease	302	1.319	5.31	$2.85 \cdot 10^{-4}$	0.029
Cognitive disorders	30	0.131	22.91	$2.88 \cdot 10^{-4}$	0.029
Colitis	31	0.135	22.17	$3.18 \cdot 10^{-4}$	0.031
Transitional cell carcinoma	33	0.144	20.82	$3.83 \cdot 10^{-4}$	0.037
Occupational asthma	7	0.031	65.44	$3.85 \cdot 10^{-4}$	0.037
Bradycardia	36	0.157	19.09	$4.97 \cdot 10^{-4}$	0.045
Common migraine/Pleurisy/Cutaneous leishmaniasis**	8	0.035	57.26	$5.12 \cdot 10^{-4}$	0.045
Thyroid neoplasm	37	0.162	18.57	$5.40 \cdot 10^{-4}$	0.047
Pneumonia	89	0.389	10.29	$5.80 \cdot 10^{-4}$	0.0495

Note. * The enrichment analysis was conducted by WebGestalt [30, 31] using the ORA (Overrepresentation Enrichment Analysis) method, for the "Diseases" category – according to DisGeNET [27, 28]; *N* – the total number of known genes associated with the disease/trait; *E* – the expected number of associated genes for a given disease/trait from those tested; *R* – excessive representation of genes in the test panel compared with the expected number (enrichment); *p* – the achieved level of significance in assessing the enrichment, *FDR* – the level of significance with the Benjamin–Hochberg adjustment. ** Diseases with the same calculated parameters are given, but which may be associated with different genes.

inflammation in the development of the above-mentioned diseases. Support for neurogenic inflammation as one of the pathogenetically significant processes in these pathologies is provided by clinical and experimental studies [2, 29, etc.].

The data given in the DisGeNET database [27, 28] on the association of genes with diseases have different evidence base, but for 18 of them highly significant connections were established (table 3). Variants of neurogenic inflammation genes not only predisposed to the development of diseases of the multi-factorial nature, but also acted as a cause of monogenic diseases. Among the diseases with the proven involvement of neurogenic inflammation genes in the pathogenesis, there is a wide range of mental and neurological disorders (vascular dementia, familial autonomic dysfunction, mental incapacity, etc.), asthma, migraine, endocrine disorders (obesity), pain sensitivity disorder, immune disorders, etc. Certainly, not all of the diseases listed in tables 2 and 3 have neurogenic inflammation as the only mechanism in the pathogenesis, but this component can be expected to be at least a modifying factor in the development and clinical picture of the disease.

Table 3

Diseases for which the pathogenetic significance of genes involved in neurogenic inflammation has been proven

Genes	Diseases
TNF	Susceptibility to asthma {600807/AD}, malaria, cerebral, {611162}, migraine without aura {157300/AD}, vascular dementia[$], septic shock[$]
IL1B	Familial Ménière disease (L); gastric cancer risk after *H. pylori* infection {137215/AD}
PTGS2	Familial Ménière disease (L)
POMC	Obesity, adrenal insufficiency, and red hair due to POMC deficiency {609734/AR}; susceptibility to obesity, early onset {601665/Mu, AR, AD}
NGF	Intellectual disability* (S); pain disorder (S); Charcot–Marie–Tooth disease (S); familial dysautonomia (S); sensory and autonomic hereditary neuropathy, type V {608654/AR}
NTRK1	Intellectual disability* (S); pain disorder (S); Charcot–Marie–Tooth disease (S); familial dysautonomia) (S); congenital insensitivity to pain with anhidrosis {256800/AR}; familial medullary thyroid carcinoma {155240/AD}
TRPV4	Charcot–Marie–Tooth disease (S); arthrogryposis (S); intellectual disability* (M); Klein–Levin syndrome (L)
DDC	Intellectual disability* (S); aromatic L-amino acid decarboxylase deficiency {608643/AR}
IL7R	Severe combined immunodeficiency, T-cell negative, B-cell/natural killer cell-positive type {608971/AR}
TRPA1	Pain disorder (S); familial episodic pain syndrome {615040/AD}
APP	Periodic fever syndrome (L); familial Alzheimer's disease, 1{104300/AD}; cerebral amyloid angiopathy, Dutch, Italian, Iowa, Flemish, Arctic variants {605714/AD}
CRLF2	Intellectual disability* (L)
IL31RA	Periodic fever syndrome (L); primary localized cutaneous amyloidosis, 2 {613955/AD}
HDC	susceptibility to the Tourette syndrome {37580/AD}
OSMR	Periodic fever syndrome (S); primary localized cutaneous amyloidosis, 1 {105250/AD}
AMN	Congenital anemia (S), cytopenia** (S); megaloblastic anemia – 1, Norwegian type {261100/AR}
TPH2	Intellectual disability* (S); susceptibility to attention deficit hyperactivity disorder, 7 {613003}; susceptibility to unipolar depression {608516}
TPSAB1	Ehlers–Dunlos syndrome (L)

Note. The level of evidence of the association between the gene and pathologies according to ClinGen and Genomics England (taken from DisGeNET [27, 28]) is given in parentheses: S is a strong link; M is a moderate bond, L is a weak bond; in curly brackets, catalog OMIM number and type of inheritance are given (AD is autosomal dominant; AR is autosomal recessive, Mu is mutational nature) [32]. The following notation is used: * groups of pathologies, ** phenotypes; [$] phenotype number in OMIM is not specified.

In addition to structural variants of genes, the course of neurogenic inflammation may be influenced by epigenetic mechanisms, which, in turn, may depend on environmental factors. Thus, between pregnant women with preeclampsia and women with normal pregnancy there are differences in the level of methylation of neurogenic inflammation genes (*POMC*, *CALCA*) in blood leukocytes [33], which can also determine differences in the level of expression of these genes. According to the information provided in DisGeNET

[27, 28], variants of these two genes are also associated with hypertension, as shown in the vast majority of associated studies performed.

COMORBIDITY OF DISEASES WITH A SIGNIFICANT COMPONENT OF NEUROGENIC INFLAMMATION IN THE PATHOGENESIS

The similarity of the genetic and biochemical components of neurogenic inflammation in various pathologies suggests the possibility of forming a comorbidity (polymorbidity). The largest number of clinical studies were carried out to investigate comorbidity between allergic and neuropsychiatric diseases (see review [34]). The obtained data strongly show that not only allergic diseases increase the probability of comorbidity with neuropsychiatric diseases, but patients with neuropsychiatric disorders have a higher risk of developing allergic pathologies. Other examples of comorbidity are also known. Thus, it has been shown that in bronchial asthma exacerbation of diseases of the gastrointestinal tract is often observed (in gastric mucosa, an increase in the number of cells activated by histamine is registered during exacerbation of bronchial asthma) [35]. In depression, dysregulation of the enzymatic production and degradation of catecholamines, neurotransmitters (including histamine), hormones, and immunological proteins is detected. Cyclic interactions are recorded between these molecules, when an increase or a decrease in one parameter can lead to stimulating or inhibitory action for others [22], which is well consistent with the model of neurogenic inflammation in which histamine is involved (Fig. 1). It has also been found that an increase in the level of inflammatory markers in brain tissues can lead to a change in the systemic immune response at the periphery [36]. In animal studies, chronic stress has been shown to increase the expression of *IL1B* and *TAC1* genes in white blood cells, as well as to disrupt the functioning of the pulmonary system [37]. In case of comorbidity of various diseases (for example, allergic and neuropsychiatric disorders), the commonality of the pathophysiological mechanisms is maintained not only by similar neurogenic inflammation mechanisms, but also by the results of genetic studies performed using both the candidate gene approach [38] and Genome Wide Association Studies (GWAS) [39].

CONCLUSION

Thus, the commonality of the pathophysiological processes in the development of neurogenic inflammation and the genetic features of individuals according to polymorphic variants of neurogenic inflammation genes may underlie the formation of comorbid conditions, such as allergic diseases, manifested at the level of various organ systems (bronchopulmonary system, gastrointestinal tract, etc.), and neuropsychiatric disorders. It can be assumed that the development of chronic neurogenic inflammation in one organ can increase the risk of comorbid inflammatory diseases in other organ systems. This can be facilitated by the transfer of histamine, substance P, and other key mediators of neurogenic inflammation (including penetration through the damaged blood-brain barrier), as well as some environmental factors with a unidirectional adverse effect (for example, excessive intake of histamine with food), especially if the genetic features of individuals are favorable for development of neurogenic inflammation.

Therefore, it is important to identify the diseases in the pathogenesis of which neurogenic inflammation plays a significant role. Establishing the commonality and specificity of the pathophysiological processes of neurogenic inflammation at biochemical and genetic levels in the development of pathological conditions of different organ systems is essential for understanding the patterns of disease formation, which may help prevent the disorders and determine the treatment strategy for patients, including the ones with comorbidities.

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Alternative risk factors and their importance in assessment of cardiovascular risk in asymptomatic patients

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ABSTRACT

Cardiovascular diseases retain their leading position among the leading causes of death worldwide. The contribution of many factors to increasing risk of developing cardiovascular diseases was proven. The article provides an overview of current views on the role of risk factors in assessment of cardiovascular risk in asymptomatic patients. Determination of individual cardiovascular risk is not questioned. However, more information is accumulating on the need to supplement the existing cardiovascular risk assessment scales with new factors in order to more accurately predict cardiovascular risk. The value of alternative risk factors, such as psychosocial factor, level of physical activity, family history of cardiovascular diseases, coronary artery calcification, ankle-brachial index, and identification of atherosclerotic plaques during ultrasound scanning of the brachiocephalic arteries, is described. Studies that consider the impact of these risk factors on reducing discrimination against cardiovascular risk when added to the globally used risk assessment scales are presented.

Key words: cardiovascular risk, subclinical atherosclerosis, coronary artery calcification.

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Нетрадиционные факторы риска и их значение в оценке сердечно-сосудистого риска у бессимптомных пациентов

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РЕЗЮМЕ

Сердечно-сосудистые заболевания (ССЗ) сохраняют лидирующие позиции среди ведущих причин смертности во всем мире. Доказан вклад многих факторов в увеличение риска развития ССЗ. Представлен обзор современных представлений о роли факторов риска у бессимптомных пациентов в оценке сердечно-сосудистого риска. Определение индивидуального сердечно-сосудистого риска не подвергается сомнению,

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однако накапливается все больше сведений о необходимости дополнения имеющихся шкал оценки сердечно-сосудистого риска новыми факторами с целью более точного его прогнозирования. Отражены сведения о значении нетрадиционных факторов риска, таких как психосоциальный фактор, уровень физической активности, семейный анамнез развития ССЗ, коронарная кальцификация, лодыжечно-плечевой индекс, выявление атеросклеротических бляшек при ультразвуковом сканировании брахиоцефальных артерий. Приведены исследования, рассматривающие влияние этих факторов риска на улучшение дискриминации сердечно-сосудистого риска при добавлении их к используемым в мире шкалам оценки риска.

Ключевые слова: сердечно-сосудистый риск, субклинический атеросклероз, коронарная кальцификация.

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

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INTRODUCTION

Atherosclerosis is an inflammatory disease with damage to the arterial wall. The development of atherosclerosis is a dynamic and multifactorial process. For a long time, atherosclerosis was considered as a disease the pathogenesis of which is associated only with the deposition of lipids in the wall of the artery. The morphological substrate of the affection of the vascular wall is well understood, yet, the mechanisms that trigger this affection are still being studied. Over the past two decades, the perception of atherosclerosis has gradually changed and was supplemented with the understanding of the role of arterial wall inflammation in the disease [1]. As a result, the leading role in the pathogenesis of atherosclerosis belongs to the lipid infiltration hypothesis and the response to damage hypothesis. Complementing one other, these concepts led to the understanding that atherosclerosis is a complex inflammatory process which involves, in particular, endothelial damage, deposition of oxLDL in the intima, proliferation of smooth muscle cells, and macrophage infiltration and activation [2, 3].

Upon progression, atherosclerosis results in such socially sensitive diseases as coronary artery disease (CAD) and cerebrovascular disease (mainly ischemic stroke). CAD and stroke are the first and third leading mortality causes worldwide, respectively. These diseases account for 247.9 deaths per 100,000 people, which accounts for 84.5% of deaths from cardiovascular diseases (CVDs) and 28.2% of deaths from all causes [4]. Other, less widespread, complications of atherosclerosis include damage to the aorta and peripheral vessels. To date, CVDs maintain a leading position among the mortality causes worldwide. In the Russian Federation, according to 2016 statistics, the mortality rate from all causes amounted to 1, 284.3 per 100,000 people, of which mortality from circulatory diseases was 616.4 per 100,000 people, and the mortality rate from CAD was 328.5 per 100, 000 people [5].

The concept of cardiovascular risk factors was introduced into medical practice more than 70 years ago. Over the past few decades, there has been an increase in CVDs around the world. This epidemiological change is partially associated with changes in lifestyle and diet; however, these factors are the main modifiable causes of CVDs [2]. According to the response to damage hypothesis, there are a lot of factors causing endothelial damage, but the most common of them are smoking, dyslipidemia, hypertension, diabetes mellitus, abdominal obesity, etc. The risk of the atherosclerotic cardiovascular disease development increases with age. The assessment of individual cardiovascular risk may be relevant for patients with asymptomatic atherosclerotic vascular disease, especially for identifying high cardiovascular risk in such patients. The assessment of individual cardiovascular risk is evaluated in general clinical practice during screening of large population groups (for example, during regular health check of certain population groups).

The tool for such assessment should be simple, reliable, convenient to be used in research and specific to the country in which it was developed and used. Many algorithms have been proposed for quick and accurate calculation of individual cardiovascular risk. In the Russian Federation, along with European

countries, the SCORE scale has been used since 2003 [6, 7]. Other well-known rating scales include Framingham risk score (FRS) (USA), Reynolds (USA), Q-RISC (UK), PROCAM (Germany), ASSIGN (Sweden), CUORE (Italy), and others [6, 7].

The SCORE scale was developed based on incidence studies in 12 European countries, including the Russian Federation. More than 200,000 patients were involved. There are 2 versions for the SCORE scale for countries with high (including the Russian Federation) and low risk. The following risk factors were included: gender, age, smoking, systolic blood pressure, and total cholesterol. The 10-year risk of developing the first fatal, associated with atherosclerosis, event – stroke, myocardial infarction, aortic aneurysm – was assessed with its help. Allowing to assess the development of lethal events in the future, this rating scale did not allow to evaluate the risk of developing non-fatal diseases associated with atherosclerosis. However, such possibility exists. To evaluate a combined risk (fatal and non-fatal), it is necessary to multiply the risk values of a particular male patient by 3 and female – by 4 [6, 7].

The findings on the association of cardiovascular diseases with various risk factors, such as arterial hypertension and hypercholesterolemia, were first obtained in the Framingham Heart Study [6, 7]. The Framingham scale was developed on its basis. This scale takes into account such risk factors as age, gender, blood pressure, smoking, the use of antihypertensive drugs, total cholesterol, and high-density lipoprotein cholesterol. This rating scale predicts the occurrence of events related to coronary artery disease in asymptomatic patients over 10 years, the endpoint being CAD (angina pectoris, myocardial infarction, sudden death).

Identification of high individual cardiovascular risk results in the need for correcting modifiable risk factors. However, this fact is important not only for high-risk patients, but also for people at low and medium risk. In 1985, D. Rose formulated the prevention paradox: more low-risk individuals may have more cases of disease than a small number of high-risk patients [7, 8]. In other words, patients at high risk have the maximum individual benefit from taking measures to control risk factors.

The individual benefit of conducting risk correction measures is not so significant among low-risk individuals, but they will bring great benefits to society on the whole due to a large number of low-risk groups. Based on the Rose paradox, the identification of cardiovascular diseases at the asymptomatic stage in patients of any risk can have a great economic importance.

With regard to the advantages of using the rating scales described above, more and more information is accumulating about their defects and limitations. Therefore, search for other risk factors is underway. The addition of new risk factors to the scales will contribute to more accurate determination of cardiovascular risk in the population groups.

Despite the fact that arterial hypertension is an independent disease, it belongs to one of the main, well-studied risk factors for CVD. In the recommendations of the European Society of Cardiology / European Society for Arterial Hypertension on Treatment of Arterial Hypertension in 2018, the influence of arterial hypertension on target organ damage was considered especially significant; it leads to an increase in cardiovascular risk, even if the damage is asymptomatic [9]. Also, in 2018, new factors which increase the risk of cardiovascular complications were identified, such as hyperuricemia, resting heart rate, diabetes mellitus, a family history of early onset hypertension, early menopause, sedentary lifestyle, and psychosocial and socio-economic factors. These factors are recommended to be considered when stratifying risk in patients with arterial hypertension [9].

Cardiovascular risk factors requiring further study include the psychosocial factor (anxiety, depression), assessment of the level of physical activity, the presence of CVD in the family history (in women – under 65 years old, in men – under 55 years old), ankle-brachial index (ABI), detection of atherosclerotic plaques during ultrasound examination of the carotid arteries, and determination of the coronary artery calcium (CAC) [6].

THE EFFECT OF STRESS ON THE DEVELOPMENT OF CORONARY EVENTS

In 2017, a group of European scientists evaluated the relationship of stress in the workplace with the following development of coronary events (the first non-fatal or fatal myocardial infarction) [10]. The study is based on the fact that stress occurs as a result of the impact of work, which is characterized by high psychological stress. A model was chosen to assess the imbalance between hard work and remuneration. Based on this theory, stress is created by a repeated unsatisfactory relationship between hard work, for example the pace of work, work load, time spent at work, and received remuneration. In addition to sala-

ry, remuneration includes non-financial aspects such as respect, recognition, career prospects, and employment opportunities. Using data from questionnaires, the imbalance between hard work and remuneration was assessed. The cohort included 90, 164 people (men and women) without CAD at the time the study began. During an average follow-up of 9.8 years, 1, 078 coronary events were recorded. As a result, it was found that in individuals with an imbalance between hard work and remuneration, the risk of CAD increased by 1.16 times (relative risk (RR) was 1.16; 95% CI 1.01 – 1.34). The relationship between unfavorable psychosocial work environment and CAD was also demonstrated.

THE EFFECT OF SUBCLINICAL CAROTID ARTERY ATHEROSCLEROSIS ON CARDIOVASCULAR RISK

The importance of detecting atherosclerotic plaques in the carotid arteries as predictors of the development of cardiovascular diseases was studied by J.F. Polak et al. in 2013 [11]. The change in the predictive value of the Framingham scale was assessed with the addition to the existing criteria of atherosclerotic plaques revealed during ultrasound scanning of the carotid arteries. The cohort study consisted of 6, 562 individuals (mean age was 61.1 years; 52.6% of patients were women). In 41.9% (n = 2, 748) of individuals included in the study, plaques narrowing the lumen by less than 25% were detected; in 13.2% (n = 863) of patients, plaques narrowing the lumen by 25% or more were revealed. CVD developed in 7.9% of the examined (n = 515). The detection of any atherosclerotic plaques was reliably associated with the incidence of CVD, with the strongest association being observed for plaques that narrowed the lumen by 25% or more (RR 1.65; 95% CI 1.34 – 2.03). Therefore, the addition of atherosclerotic plaques of the carotid arteries to traditional risk factors more accurately predicts the possibility of CVD development and improves the prognosis of cardiovascular risk.

The value of coronary artery calcification

Some researchers believe that the detection of coronary artery calcification is actually pathognomonic of atherosclerosis of the coronary arteries [3]. At the same time, calcium phosphate in hydroxyapatite form accumulates in the intima affected by atherosclerosis. The calcification of the atherosclerotic plaque begins in the lipid nucleus of the atheroma and occurs as an active process resembling bone formation, controlled by complex enzymatic and cellular pathways. The underlying mechanisms are not completely understood, but apoptosis of smooth muscle cells, apparently, is an important stage in this process, which then serves as a focus of calcification. Based on histomorphometric studies, approximately 20% of atherosclerotic plaques of the coronary vessels are calcified and these macrocalcifications can be identified using non-contrast computed tomography [2, 3].

In 2008, Detrano et al. for the first time reported about the relationship between the level of CAC and following coronary events (myocardial infarction or death from coronary artery disease). Based on the results of their work, researchers concluded that adding CAC to standard risk factors improves the prognosis of following coronary events [12].

ASSOCIATIONS OF CAC WITH NEW MARKERS OF ATHEROSCLEROSIS

In the study by J.A. Delaney et al. the relationship between the level of physical activity, CAC and ABI was assessed [13]. The study included individuals with an ABI from 0.90 to 1.40 (n = 5, 656), 53% of them were women with an average age of 61 years and an average body mass index of 28 kg/m^2. At the beginning of the study, approximately 33% of individuals had dyslipidemia, 11% had diabetes, and 42% had arterial hypertension, but they did not have clinically severe coronary artery disease. Slightly more than 62% of patients reported that they were engaged in intense physical activity, and 35% – in moderate physical activity. It was found that more intense exercise reduced the risk of peripheral arterial atherosclerosis (RR 0.85; 95% CI 0.74 – 0.98), and a significant relationship was identified between the intensity of physical activity and the detection rate of coronary artery calcification (RR 0.97; 95% CI 0.94 – 1.00). In general, a reliable relationship between the intensity of physical activity and an increase in CAC was not detected, however, sedentary lifestyle was largely associated with an increase in CAC (Δ log (Agatston score +25) = 0.027; 95% CI 0.002 – 0.052). Therefore, sedentary lifestyle was associated with the progression of damage to peripheral and coronary arteries. This study showed a significant role of moderate and intense physical exertion in preventing the progression of atherosclerosis of peripheral arteries. With regard to coronary artery calcification, a similar fact was not detected, however, it was determined that any physical activity reduces its progression, and sedentary lifestyle increases it. As a result, any type of activity is better than sedentary lifestyle.

FAMILY HISTORY OF CARDIOVASCULAR DISEASE AND PROGRESSION OF CORONARY ARTERY CALCIFICATION

It has now been established that a zero CAC score is associated with a very low 10-year risk of cardiac events, but this risk is not equal to zero. In the 2014 study, an assessment of the family history of coronary artery disease was performed [14]. The purpose of this study was to evaluate the role of family history in the development of CVD in people with zero CAC. Higher incidence of subclinical atherosclerosis in individuals with a family history of CAD, as opposed to individuals without it, was established. The work included 3, 185 individuals with basic assessment of CAC = 0 (average age 58 years, 37% of men). The average risk score according to the Framingham scale was 6.1% for individuals with a family history of CAD and 6.2% for people without it ($p = 0.84$). On average, over 10 years, 101 (3.2%) individuals had CVD, and 56 (1.8%) had episodes of CAD. In analyzing age and gender, a family history of CAD was associated with an increase in CVD cases by approximately 1.73 times (RR 1.73; 95% CI 1.17 – 2.56) and with CAD (RR 1.72; 95% CI 1.01 – 2.91). The researchers concluded that asymptomatic individuals with zero CAC and a positive family history of CAD were at increased risk of cardiovascular and CAD events in comparison with those who did not have a family history of CAD, although absolute incidence rates remained low.

A similar study was conducted a year earlier [15]. It was demonstrated that a family history of early CAD was usually associated with the progression of coronary artery calcification among asymptomatic individuals. Of the total cohort, 47% were men. Overall, 52% ($n = 2, 633$) of individuals had a positive family history of CAD; 20% ($n = 1, 002$) of people had a family history of early CAD, of which 456 reported a similar history only with their parents, 471 – only with brothers and sisters, and 75 – with parents, brothers, and sisters. The average risk score according to the Framingham scale was 7.5% ($n = 2, 466$) for individuals without a family history of CAD, 8.2% ($n = 1, 631$) for people with a family history of late-onset CAD, and 7% ($n = 1, 002$) for people with a family history of early CAD. In the main group, 2, 645 people (52%) did not have coronary artery calcification at the beginning of the study. Among them, in 527 (20%) patients, CAC became higher than zero during the following examination. A significant increase in CAC was observed in patients with a family history of early CAD (7.24 per 100 person–years) compared with patients without a family history of CAD (5.87 per 100 person–years) or with a late-onset CAD in the family history (6.56 per 100 person–years) ($p < 0.05$). In individuals with a family history of early CAD, the increase in CAC was 16.7 units higher than in individuals without it ($p < 0.001$). This was more than twice as much as in the group of individuals with a family history of late CAD (8.17 units). The obtained results confirm the opinion that a family history is an important component of cardiovascular risk and suggest that accelerated coronary artery calcification in subclinical atherosclerosis may contribute to increased risk of the disease.

THE EFFECT OF NEW CARDIOVASCULAR RISK FACTORS ON RISK DISCRIMINATION ACCORDING TO THE FRAMINGHAM SCALE

In 2016, J. Yeboah et al. published the results of the study that examined the addition of new cardiovascular risk factors to traditional ones [16]. The role of factors, such as coronary artery calcification, level of C-reactive protein, ABI, and the presence of CAD in the family history, and their significance in stratification of cardiovascular risk were assessed. It should be noted that these factors were not evaluated in aggregate, but separately. The endpoints were myocardial infarction, death from CAD, or stroke. The analysis included 5, 185 individuals; the average age was 61 years; 53.1% were women. The average follow-up period was 10 year; during this time, 320 (6.2%) cases of cardiovascular diseases were registered, of which 139 (43.4%) were cases of myocardial infarction, 132 (41.3%) – strokes, and 49 (15.3%) – death from coronary artery disease. In terms of predicting the development of CAD, CAC was the only marker that significantly improved risk discrimination according to the Framingham scale (Net Reclassification Index (NRI) 0.178; 95% CI 0.080–0.256). The addition of ABI to the Framingham model improved stratification of patients less significantly (NRI 0.013; 95% CI 0.034–0.051). A total of 194 (3.7%) cases of CAD occurred. Therefore, among the four risk markers included in the analysis, CAC showed the greatest improvement in the discrimination of risk of CAD and reclassification of patients with intermediate risk according to the Framingham scale. In addition, the authors pointed out that CAC, compared with 3 other non-traditional risk markers, is more suitable not only for improving the prognosis of the risk for CAD, but may be useful for people who have not made a decision on treat-

ment based on the risk determined by the Framingham scale. Therefore, further research is needed to improve the discrimination provided by these additional risk markers in subgroups of asymptomatic individuals (primary prophylaxis), especially those who are not recommended for statin therapy.

CONCLUSION

Despite the fact that the concept of cardiovascular risk factors was introduced into clinical practice more than seventy years ago, it is still relevant today. Most significant factors of cardiovascular risk are modifiable and have enormous prognostic value. Many studies have been conducted to determine the effectiveness of existing scales for assessing cardiovascular risk. However, despite their effectiveness, they require additions and refinements. Analyzing the results of the above mentioned studies, we can definitely say that today the problem of a correct assessment of cardiovascular risk remains relevant for all groups of patients at any cardiovascular risk. An increase in cardiovascular risk in asymptomatic patients is often associated with the psychosocial factor or detection of atherosclerotic plaques during an ultrasound examination of the brachiocephalic arteries. The role of CAC has yet to be studied; however, the studies presented in this review article established mutual potentiation of risk factors, such as a family history of coronary artery disease, sedentary lifestyle, and assessment of ABI and CAC. The study of new cardiovascular risk factors in asymptomatic population shows its discriminatory and prognostic value, but requires following detailed study to develop the most convenient, accurate and effective rating scales applicable to any category of patients, including asymptomatic ones. The development of these risk scales, according to the Rose paradox, will be of great economic and social importance.

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Neuroimaging methods for assessing the brain in diabetes mellitus (literature review)

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ABSTRACT

Diabetes mellitus (DM) is associated with changes in the structure of the brain and deterioration of cognitive functions from mild to moderate according to neuropsychological testing. With the growing DM epidemic and the increasing number of people living to old age, cognitive dysfunctions associated with DM can have serious consequences for the future of public and practical health. Chronic hyperglycemia, severe episodes of hypoglycemia, and microvascular complications are important risk factors common for type 1 and type 2 diabetes. DM is also associated with structural and functional changes in the brain, which can be diagnosed by various types of magnetic resonance imaging (MRI) of the brain.

In this review, we investigate studies conducted over the past two decades to improve the understanding of how DM effects the brain function and structure. We also describe the changes characteristic of type 1 and type 2 diabetes during standard MRI, functional MRI and proton magnetic-resonance spectroscopy (proton MRS) as well as their features.

Key words: diabetes mellitus, cognitive impairment, neuroimaging techniques.

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Нейровизуализационные методики оценки головного мозга при сахарном диабете (литературный обзор)

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РЕЗЮМЕ

Сахарный диабет (СД) связан с изменениями в структуре головного мозга и ухудшением когнитивных функций от легкой до умеренной степени по данным нейропсихологического тестирования. В условиях растущей эпидемии СД и увеличения числа людей, доживающих до старости, когнитивная дисфункция,

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ассоциированная с СД, может иметь серьезные последствия для будущего общественного и практического здравоохранения. Хроническая гипергликемия, тяжелые эпизоды гипогликемии и микрососудистые осложнения являются важными факторами риска, общими для СД 1- и 2-го типа. Также СД связан со структурными и функциональными изменениями в головном мозге, которые возможно диагностировать посредством различных вариантов магнитно-резонансной томографии (МРТ) головного мозга.

В представленном обзоре рассмотрены исследования, проведенные за последние два десятилетия, чтобы улучшить понимание того, как СД влияет на функцию и структуру головного мозга. Также опишем изменения, характерные для СД 1- и 2-го типа при проведении стандартной, функциональной МРТ и протонной магнитно-резонансной спектроскопии, и их особенности.

Ключевые слова: сахарный диабет, когнитивные нарушения, нейровизуализационные методики.

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

Diabetes mellitus (DM) is a chronic metabolic disorder that is characterized by absolute insulin deficiency in type 1 diabetes or its relative insufficiency or resistance in type 2 diabetes [1]. This is a serious problem that leads to development of complications in the peripheral and central nervous system [2]. In DM, there is a 20–70% decrease in cognitive abilities compared to healthy people, and the risk of dementia is 5% higher than in healthy people [3]. Cells and their extracellular matrix have a dynamic and reciprocal relationship. Modular components upon activation of the glycation process lead to altered neurogenesis, hyperphosphorylation of intracellular signal molecules, and expression of extracellular protein matrix. All these cellular changes can contribute to cognitive dysfunction in DM [4]. There are various methods for assessing cognitive dysfunction, such as neurocognitive testing, evoked potentials, electroencephalic research, MRI, and positron emission tomography [5].

For the most accurate diagnosis of cognitive impairment in diabetes, the method of standard brain MRI was used in practice, which allows to detect mainly macrostructural changes associated with cerebrovascular diseases, such as hyper-intensive activity of white substances and indirect signs of atrophy of brain substances [6]. The functional MRI focuses on changes in activation models, functional connectivity and signal fluctuations, as well as on interconnected cognitive impairment and activation domain names, default networks, and functional conjunctivity [7]. Proton magnetic resonance spectroscopy (PMRS) is an analytical method that allows scientists to identify and quantify metabolites in various areas of the brain and to determine energy metabolism and processes in brain tissue non-invasively [8].

FEATURES OF COGNITIVE IMPAIRMENT IN TYPE 1 DIABETES

In patients with type 1 diabetes, frequent variability of glycaemia, hyper/hypoglycemia, and cumulative chronic hyperglycemic exposure lead to such microvascular damages to organs as retinopathy and nephropathy [9]. In addition to microvascular complications, type 1 diabetes is associated with an increased risk of cognitive impairment, which primarily represents a decrease in the processing speed of information, attention, and executive function [10–12]. Cognitive dysfunction can be observed quite early (already two years after diagnosis) and persists in adulthood and at a later age [13, 14]. However, the exact neuropathological mechanism of cognitive impairment caused by type 1 diabetes is still largely unclear.

NEUROIMAGING TECHNIQUES FOR BRAIN NEUROPLASTICITY EVALUATION IN TYPE 1 DIABETES

Neuroimaging methods were used to study the anatomical and functional changes in the brain of patients with type 1 diabetes. A standard MRI scan reveals atrophy of the gray matter and lesions of the white matter, which are common structural abnormalities observed in

the studies and associated with a cognitive decline in patients with type 1 diabetes [15–17]. The decrease in the volume of the brain in the cortical and subcortical areas, including the occipital, lower frontal and parahippocampal regions, is mainly determined [18, 19]. There are no significant differences in the volume of gray or white matter of the brain compared with the control group in the article of Perantie et al. on analyzing MRI scans in children with type 1 diabetes [20]. It was shown that medical history of severe hypoglycemia is associated with a smaller volume of gray matter in the upper left temporal region, while chronic hyperglycemia is associated with a change in the volume of the gray matter in the right posterior parietal region and right prefrontal region [21]. In addition, there is evidence that these changes are noted within a few years from the start of manifestations and associated with cognitive functions [22].

Diffuse tensor MRI reveals fractional anisotropy in the upper parietal lobe and a decrease in average diffusion in the thalamus [19]. In addition, there is a decrease in fractional anisotropy in the posterior parts of the brain, which is associated with a longer duration of the disease, as well as a decrease in a number of cognitive functions, such as speed of information processing and executive functioning [23]. Using only functional MRI of the brain, van Duinkerken et al. demonstrated impaired functional conjunctivity and network changes in patients with type 1 diabetes [24]. In addition, abnormal functional conjunctivity was found in the subgenual cingulate gyrus, which was associated with cognitive dysfunction in patients with type 1 diabetes [25]. Moreover, using an analysis of independent components, it was found that type 1 diabetes is associated with a violation in several networks, including attention, working memory, hearing, language, and processing [26–28]. Since the effect of hyperglycemia on the brain can be global, an analysis of the neural function of the entire brain is likely to reveal other deficits in the central nervous system associated with type 1 diabetes.

In the studies by Mangia and Heikkilä et al., a decrease in N-acetylaspratate metabolites was shown in the gray matter (occipital lobe, frontal lobe), white matter, and thalamic regions of patients with type 1 diabetes compared with the control group [29, 30]. Besides, it was shown that patients with high glycated hemoglobin have a decrease in glucose in the brain by almost 10%. This neurochemical process can explain the loss of neurons associated with cognitive impairment [31]. In addition, there is a change in the ratio of metabolites N-acetyl aspartate/creatine, choline/creatine and N-acetyl aspartate/creatine in the left posterior parietal region of the white matter in type 1 diabetes [32].

FEATURES OF COGNITIVE IMPAIRMENT IN TYPE 2 DIABETES

Several studies have shown that type 2 diabetes (at least 90%) is a risk factor for dementia [33, 34]. Typically, patients with type 2 diabetes have a moderate decrease in cognitive functions, and the metabolic syndrome is believed to make a significant contribution to their decline [35]. Type 2 diabetes is usually diagnosed at an older age and is usually associated with obesity, insulin resistance, hypertension, and dyslipidemia, which can have a negative effect on the brain [36].

NEUROIMAGING TECHNIQUES FOR BRAIN NEUROPLASTICITY EVALUATION IN TYPE 2 DIABETES

Type 2 diabetes is associated with diffuse atrophy of the brain [37]. A decrease in the average total brain volume is more pronounced in type 2 diabetes, which is comparable with 3–5 years of normal aging [38]. Brain atrophy associated with type 2 diabetes is most pronounced in areas which are surrounding the ventricles, such as the subcortical region of the gray or white matter [39].

In patients with type 2 diabetes, there is a decrease in the functional relationship between the areas including the medial frontal gyrus, precuneus and medial temporal gyrus, which are associated with cognitive functions [40].

When performing proton MRS in patients with type 2 diabetes, a low level of N-acetyl aspartate was recorded in the right frontal and parietal–temporal regions, and glucose levels were elevated in all areas of the brain [41]. Besides, reduced levels of choline and creatine in the lenticular nuclei and areas of the thalamus and decreased N-acetyl aspartate/creatine and choline/creatine ratios were identified. These changes had a negative correlation with the level of glycaemia and glycated hemoglobin [42].

CONCLUSION

Typical signs of brain atrophy which can be detected during standard MRI are to a larger extent associated with metabolic disorders, but give no evidence of a connection with cognitive impairment and do not provide a further diagnostic algorithm [43].

In diabetes, there is a change in the spontaneous activity of the brain, especially in the visual zones, as well as a change in the functional connection in various default networks. However, the plasticity of the nervous system at a young age is possible, and the functional relationships are improved after rehabilitation measures [44].

A change in the level of N-acetyl aspartate is associated with the density, function or viability of neurons that can be found when conducting PMRS [45]. Choline concentration changes with damage to the cell membrane [46]. Creatine is involved in energy metabolism, and its increased level means increased oxidative stress and mitochondrial dysfunction, both in neurons and in glial cells [47].

Structural and metabolic changes which were described in this article lead to impaired neurotransmission, accelerated neurodegeneration and demyelination, as well as cause brain atrophy in diabetes. However, further studies should confirm the above stated results in larger clinical trials.

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Optogenetic methods and technologies in solving applied medical problems

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ABSTRACT

Optogenetics is an innovative and fast-growing field of science combining the advances in molecular biology and laser technologies to monitor various biochemical processes in the cell and to control its activity using light. Therefore, this review is devoted to the implementation of the optogenetic approach to diagnosis and treatment of various socially sensitive diseases at the molecular and genetic level. Furthermore, the article considers different methods of delivery and incorporation of genetic constructs encoding transmembrane proteins. New fiber optic technologies used to develop implantable devices for generating and recording signals in excitable tissues are described. Besides, the most state-of-the-art and popular registration methods are considered in the review.

Key words: optogenetics, opsins, ion channels, fiber optic systems, photostimulation, neurointerface, optogenetic therapy.

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Оптогенетические методы и технологии в решении прикладных медицинских задач

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РЕЗЮМЕ

Оптогенетика – инновационное и быстро развивающиеся научное направление, объединяющее достижения молекулярной биологии и лазерных технологий, для решения вопросов мониторинга различных биохимических процессов в клетке и контроля ее активности с помощью света. Данный обзор посвящен вопросам реализации и применения оптогенетического подхода для диагностики и лечения различных социально значимых заболеваний на молекулярно-генетическом уровне. В статье описаны различные способы доставки и встраивания генетических конструкций, кодирующих трансмембранные белки. Рассматриваются новые оптоволоконные технологии, используемые для исполнения имплантируемых устройств генерации и фиксирования сигналов в возбудимых тканях. Приводится анализ современных, наиболее используемых способов регистрации показателей эксперимента, указываются ключевые преимущества и недостатки различных методик.

Ключевые слова: оптогенетика, опсины, ионные каналы, оптоволоконные системы, фотостимуляция, нейроинтерфейс, оптогенетическая терапия.

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INTRODUCTION

Currently, a new interdisciplinary science – optogenetics – is rapidly developing at the intersection of molecular biology and advanced laser technologies. Researchers in this area are developing a wide variety of methods for studying the mechanisms of memory and behavior formation as well as for functional diagnosis and therapy of neurodegenerative, psychogenic and other socially sensitive human diseases. In solving such complex and complicated problems, the knowledge about the functioning of various genetic constructs and implementation of fundamentally new optical devices to study the functioning of excitable tissues is of great importance.

The latest achievements in optogenetics which have been developed and implemented in practical healthcare are based on the use of genetically encoded light-sensitive ion channels which are subsequently exposed to photostimulation in various modes. In such experiments, it is particularly important to ensure the availability of a high-quality fiber optic system which will deliver the light beam with minimal losses and effectively record intracellular changes. These systems represent a unique platform for developing innovative neural interfaces which are used in optogenetics for experiments on freely moving animals.

Currently, optogenetics has profound advantages over traditional electrophysiological methods due to its selective effect, accuracy and a possibility of both excitation and suppression of the selected cell populations. The latter can be used not only in fundamental neurobiology, but also in applied medicine. For example, it is possible to stop and/or prevent an epileptic seizure by inserting actuators into neurons in the seizure focus and enabling inhibition.

This review article is the first one to comprehensively cover the issues of planning an optogenetic experiment with specifying the key advantages and disadvantages of various methods. It also most fully describes modern advances in optogenetics used in clinical medicine.

OPTOGENETICS: THE HISTORY OF ITS DISCOVERY AND FORMATION

In 1971, W.Stockenius and D.Osterhelt discovered that bacteriorhodopsin in the ion channel can be activated by photons. After eight years, Francis Krikvy, an English biophysicist, suggested the idea of activating a group of cells with the use of light [1, 2].

In 2005, a group of scientists from Stanford University led by Karl Deisserot showed that the activity of a group of neurons can be controlled by adapting natural channelrhodopsin (Channelrhodopsin-2), obtained from green algae of the Chlamydomonas Reinhardtii

species, using lentiviral vectors for gene delivery [3]. The experiment was performed on fruit flies and mice several times. As a result, it was reliably proved that after incorporation of an opsin in the plasmalemma of a cell and its exposure to the blue spectrum light, the cell membrane was depolarized.

Further studies showed that other natural proteins, such as bacteriorhodopsin, halorhodopsin, and channelrodopsin, are also capable of regulating the functioning of neurons in response to the light irradiation of different wavelengths. In 2008, Volvox channelrhodopsin-1 (VChR1) was isolated for the first time, and it was already sensitive to the yellow spectrum light [4]. This discovery demonstrated that the use of various modifications of channelrhodopsins with activation peaks shifted to the red spectrum area allows the researcher to stimulate selectively two types of neurons located in the same area of interest.

With updating the research in this area and obtaining new knowledge about the molecular organization of the brain, it became clear that the tissues of the vertebrates already contain trans-retinal which is necessary for the implementation of the method. By absorbing a photon, the retinal isomerizes, thus provoking a change in the protein conformation, which results in a change in the membrane permeability for ions, inducing the current of monovalent (H+, Na+, K+) and some divalent (Ca2+) cations, which, in turn, cause depolarization of the neuron membrane. Now researchers can selectively control the activity in certain neurons, as well as predict physiological and behavioral responses of organisms.

Introduction of new research methods in the field of neurobiology enabled to improve fiber optic tools which allow for the light beam delivery. Thus, the idea of simultaneous optical stimulation and registration of electrical impulses has been successfully implemented. For example, currently it is possible to directly measure the electrical activity in neurons which are responsible for motor activity and to simultaneously control them with the use of opsins.

AN OPTOGENETIC STUDY: PRINCIPLES OF PLANNING AND DEVELOPING THE EXPERIMENT DESIGN

The design of any optogenetic study includes the following main elements:

Planning of an experiment

At this stage, the aim and objectives of the experiment are set, the conditions of the experiment are specified (available or accessible equipment, financial resources, personnel), the input and output parameters are identified on the basis of the collected and analyzed preliminary information (determining the object of the study, the method of delivery of the genetic construct encoding the light-sensitive protein, and the mode of photostimulation), and the plan and timing of the experiment are set as well.

Delivery of the genetic construct encoding the light-sensitive protein

Currently, the method of injecting adeno-associated virus (AAV) and lentiviral (LV) vectors is widely used, which allows to have accurate spatial control over the opsin expression. The specificity of the method is mainly achieved through the use of promoters and enhancers. Only in cells with the corresponding expression pattern for a particular promoter can opsin synthesis be activated [5]. The use of enhancers makes it possible to achieve the strict specificity without overloading the vector design [6].

A set of receptor proteins on the surface of the viral capsid called serotype plays a significant role in the effectiveness of the implementation of viral particles, since the serotype determines in which way the target cells will be infected (for example, in the area of the neuron body or at its processes). It has already been experimentally proved that the AAV 2.1 serotype is suitable for infecting rodent brain cells, and the serotypes 8 and 9 are suitable for infecting neurons in primate brain.

Another strategy that ensures the presence of a specific gene in the experiment is the use of transgenic animals, i.e. living organisms in whose genome foreign genes were introduced. Currently, cre / loxP animal lines are widely used, that express cre recombinase, which excises the exon surrounded by LoxP sequences. As a result, an animal line is created which misses a certain gene that is of interest for researchers [7].

To create transgenic animals, viral and non-viral technologies are used. The latter include approaches based on physical and chemical effects that allow for cell transfection *in vitro* [8]. One of the methods of creating such transgenic research objects is associated with embryonic stem cells. First, the cloned DNA is integrated into the embryonic stem cell culture, then the selected transgenic embryonic stem cells are cultured and used to create the necessary lines [9, 10].

To obtain transgenic mice, the method of intraportal electroporation is also used. In this case, a solution with DNA encoding opsin is injected *in utero* on

certain days of embryonic development, and then it is exposed to a short high-voltage electric discharge, which allows to impact selectively on specific cell types and brain regions [11]. This method requires exact knowledge of the time and migration trajectory of certain neuron groups during embryonic development. This technique allows to provide high quality targeted gene delivery to the II and III cortical layers, striatum and hippocampal neurons [12]. Unlike viral methods, electroporation can introduce more gene copies and deliver DNA of any size with large promoter segments to achieve higher cell specificity.

The most well-known chemical method of genetic construct delivery is liposome transport: the genetic material is placed in a liposome, which binds to the cell membrane and promotes its intracellular release. The specificity of this method is achieved by binding a specific ligand to the liposome surface.

So, currently, there is a wide range of methods that provide the presence of opsin-encoding genes in experimental models. The priority characteristics of the above mentioned methods are precision and selectivity of action, relative accessibility, and reproducibility. The most common method for optogenetic studies is the use of transgenic animals and viral vectors. However, development and implementation of new methods for genetic construct delivery are also relevant, since they will significantly expand the scope of optogenetics and accelerate implementation of the method into practice, including its use for human treatment.

Light beam delivery

In general, the functions of the optical fiber complex for light delivery are as follows: generation of and control over light emission, modification of light emission and its filtering, and transport of light to tissues and cell cultures [13]. Today, even the simplest optogenetic experiments require programmable pulse generators to modulate LED or laser emission and create a high-quality controlled light impulse. Studying the brain functions requires neural interfaces that can record the brain activity and stimulate it with high precision in space and time. Among researchers, the most popular method is *in vivo* optogenetic technique in the laboratory, for which implantable optical fiber is permanently inserted and guided through the cannula. Fiber implantation overcomes such limitations as brain tissue damage during second insertion of fiber, potential fiber defects within the implant, and accuracy of cannula positioning inside the animal brain. One of the advantages of the permanent optical fiber implantation in *in vivo* experiments is the possibility of combining the optogenetic method with other types of investigation. The main parameters that must be determined before starting any optogenetic experiment are the wavelength, the intensity and mode of stimulation, which determine the success of the study and intracellular activity management.

Registration of the results of the optogenetic experiment

To visualize changes in the parameters of cellular activity in freely moving animals, a large number of different biosensors can be used, including genetically encoded ones based on the fluorescence effect. These methods make it possible to evaluate not only cellular activity, but also a change of the cell signal status. For example, the family of GACh acetylcholine sensors based on G-protein receptors that selectively respond to a specific mediator with fluorescence recorded by epifluorescence, confocal, and two-photon microscopy is widely used [14]. Scientists are interested in the possibility to manipulate a certain neuron population and simultaneously register the results, which will allow to study the relationship between cellular activity and cellular functions in more detail. The latest development in this area is a miniscope, which allows to visualize fluorescence and make simultaneous optogenetic manipulations, changing the activity of the neurons [15].

One of the most common methods for assessing the electrophysiological changes in the membrane potential of excitable cells is the patch clamp technique. The use of this method *in vitro* in combination with optogenetics makes it possible to investigate the synaptic activity of isolated neurons and establish their role in activating and inhibitory effects on the cerebral cortex.

Behavioral tests are used to assess neurological status and monitor animal behavior

Thus, the diversity of modern optogenetic technologies determines the possibility of their application as a main or additional method in various fields of science. However, further development and improvement of optogenetic technologies remain relevant. The development of biotechnology, genetics, optics, and biochemistry allows to fully realize the potential of these research areas. In particular, at each stage of the optogenetic study, several challenges can be distinguished. At the first stage, improvement of methods of gene delivery and expression is relevant, as well as a targeted study of genes encoding photosensitive pro-

teins, which is especially important for future use of optogenetics for treatment of human diseases. At the second stage, it is important to further study the well-known photosensitive proteins: not only their physicochemical properties, but also their functions in the cells of the organisms in which they were found. This will allow to further consider them in the pharmacological aspect. Of particular interest is the possibility of modifying photosensitive proteins, due to which it will be possible to finely regulate their functions in the optogenetic experiment. Studies at the third stage are aimed, firstly, at investigating modified light irradiation for developing new methods using light of different wavelengths, focused, scattered, and multipath irradiation. Secondly, they are aimed at improving the hardware complex, which is especially important in order to standardize the method. Finally, at the fourth stage, the problem of an objective evaluation of the experimental results arises, to overcome which a comprehensive analysis is required, including functional tests, registration with electrodes and probes, and fluorescence biosensors. It is also essential to develop non-invasive methods for recording results, especially in the field of medicine.

APPLICATION PROSPECTS: OPTOGENETIC APPROACH IN THE SOLUTION OF BIOMEDICAL TASKS

Neurophysiology

Optogenetic selectively impacts on excitable tissues and has a number of advantages in comparison with other methods studying this fundamental area (in particular, in comparison with classical neurostimulation). The advantages of optogenetics include the ability to influence certain types of neurons of interest and their individual intracellular structures with precise spatiotemporal control. This determines the prospects for expanding modern ideas about the functional structure of the brain. Thus, the possibility of activating locomotion movements by light stimulation of glutamatergic neurons of the spinal cord in transgenic mice was experimentally demonstrated, which showed the key role of these cells in the process of movement [16].

Optogenetics may also be the key to solving more complex, integrated research tasks. For example, by supplementing the experiment with functional data using the optogenetic method, it was found that neurons of the rostral ventromedial medulla oblongata innervate functionally heterogeneous tissues (myocardium, skeletal muscle). Previously it was suggested that this brain region is associated with the regulation of the tone of smooth myocytes alone [17].

In addition, the possibility of studying the cerebellum using the optogenetic approach is discussed, which is especially relevant considering the poorly studied functional connections of the cerebellum with the cerebral cortex and subcortical structures [18].

Neurology

The mentioned advantages of optogenetics determine its wide range of possibilities not only in the field of neurophysiology, but also in clinical disciplines, such as neurology and psychiatry, making it possible to consider this approach as a promising treatment method. One of the areas of optogenetic research is the possibility of treating neuropathic pain. The exact pathogenesis of this condition is unknown. However, the possibility of effecting its peripheral and central components was experimentally proved. In the first case, nociceptors were considered a therapeutic target, in the second – a gelatinous substance [19].

In an experiment with a model of Alzheimer's disease it was possible to establish the therapeutic role of restoring slow oscillations (0.6 Hz) in the corticothalamic networks by optogenetically increasing the activity of pyramidal neurons. Moreover, the pathogenic effect of slow oscillations in the corticothalamic networks with optogenetically doubled frequency (1.2 Hz) on the development of this pathology was identified. This creates the prerequisites for the development of medical and preventive care based on the achievements in optogenetics [20].

Since 2010, scientists have begun to propose methods of treatment for Parkinson's disease through deep brain stimulation. The method is based on the stereotactic use of miniature electrodes that stimulate the subthalamic nucleus of the brain. The effectiveness of using this treatment option far exceeds the effectiveness of drug therapy.

A functional optogenetic approach is also considered as a rehabilitation method, in particular, after cerebral infarction. In this case, a multicomponent action is possible: an increase in neuronal activity in ischemic tissues in combination with the reorganization of afferent and efferent neural pathways [21].

Psychiatry

The prospect of functional reorganization of afferent and efferent neural pathways, and, in particular, a targeted study of cellular and subcellular interactions in the nervous tissue determine a great interest of psy-

chiatrists and narcologists in optogenetics.

It is assumed that by optogenetic normalization of biochemical processes and selective stimulation of brain regions pathogenetically associated with a particular disease it will be possible to treat diseases with depressive syndrome, anxiety, addiction, as well as schizophrenia and autism spectrum disorders [22, 23]. In particular, in one of the experiments, American scientists identified a significant decrease in depressive symptoms in animals after optogenetic stimulation of dopaminergic neurons associated with the nucleus accumbens, responsible for the formation of various behavioral reactions [24].

In 2014, scientists from the University of Buffalo managed to control addictive dependence in rats habituated to alcohol using light. In this group of animals, genetic modification of dopamine release systems was carried out, thereafter, with the help of light it was possible to stimulate selected groups of neurons and achieve prolonged release of the neurotransmitter [25].

In another experiment using optogenetic stimulation of the orbitofrontal cortex in experimental animals, inhibition of compulsive symptoms was detected, which creates the prerequisites for the development of treatment for a number of disorders associated with impaired interaction between the orbitofrontal cortex and striatum [26].

Ophthalmology

The ability to restore and regulate photosensitive cells using optogenetic stimulation determines the prospects for using this method in the field of ophthalmology, including treatment of retinal diseases, which is extremely important given the disappointing WHO statistics on morbidity of retinal degenerative diseases.

It should be noted that optogenetic studies conducted in the field of ophthalmology have a number of differences compared to other areas of medical research. First of all, opsins with a retinoid cofactor are most preferred, which is more physiologically reasonable; and, secondly, genetic material is usually delivered with viral vector injections in the intraretinal and subretinal space using an adeno-associated virus [27]. The main problems of application of the optogenetic method in this area include the occurrence of retinal remodeling with impairment of cytoarchitectonics and functional relationships between its layers, problems with precise determination of the site for the required injection to obtain an optimal result, and some mismatch between physiological ranges of light perception and ranges of light to which opsins react [28]. However, it is assumed that continuous improvement of the optogenetic experiment technique will overcome the difficulties encountered. Thus, nowadays, optogenetic engineering technologies allow to sensitize to light not degraded photoreceptor cells, but ganglion cells, which reduces the risk of developing complete loss of vision in patients with retinal degenerative diseases [29].

Otorhinolaryngology

Optogenetics also opens up wide opportunities in the field of otorhinolaryngology. In particular, this approach can be used to restore hearing impairment at the receptor level when acting on physiological mechanisms of sound perception, as was shown in experiments with optogenetic stimulation of the auditory nerve, as a result of which excitation of the corresponding nuclei of the brain stem was recorded [30, 31]. Obvious advantages distinguish optogenetic constructs from cochlear implants, allowing to impact selectively on cells in a certain part of the cochlea. This experiment was carried out by researchers from the University of Massachusetts, and they achieved partial restoration of hearing using low-intensity light [32].

Endocrinology

Optogenetic stimulation may be used for correcting the pathogenesis of endocrine diseases and developing a blood glucose lowering system in the long run. The possibility of achieving normoglycemia in a model of type II diabetes mellitus has been experimentally established. During the study, a cell culture secreting glucagon-like peptide-1 (GLP-1) and alkaline phosphatase was transplanted into LepRdb/db mice intraperitoneally and subcutaneously. In the first case, the light beam was supplied using optical fiber, in the second case, it was delivered transdermally; and in both cases, a significant decrease in blood glucose was registered [33]. A similar logic of the experiment was also preserved in a study with wireless control over the process of supplying a light beam using a smartphone app. Moreover, the cell culture also synthesized GLP-1 and insulin, and a subcutaneously implanted LED with the culture in a hydrogel capsule was used as a light source [34].

Cardiology

Due to the fact that the functions of the heart are inextricably linked with electrochemical processes, optogenetics opens up great opportunities for recovery of patients with cardiovascular diseases, since it

targets more physiological and subtle mechanisms than currently used medical and surgical methods of treatment.

The advances in optogenetics can be used to obtain a new type of pacemaker in which the function of cardiac pacemaker cells will be controlled by light instead of electrical impulses. For example, in the United States, a team of Stanford scientists led by Oscar Abilez is working on a project to develop a new biological pacemaker controlled by light. The results of the studies suggest that there are reliable ways to restore the healthy functioning of the heart muscle using light. The most important advantage of optogenetics in cardiology is selective excitation of only the inner layer – the endocardium [35]. This method of treatment of atrial fibrillation will improve the condition of patients and reduce side effects compared to the use of existing electric pacemakers, which eliminate arrhythmia, but cause severe pain due to excitation of skeletal muscles [36, 37]. The optogenetic method can currently replace devices, such as pacemakers and defibrillators, which allow to deliver electrical signals at a certain rhythm, but carry certain risks (damage to the heart tissue, battery failure, etc.) [38].

Pharmacy and pharmacology

No less relevant is the use of optogenetics in the field of pharmaceutical sciences. First of all, optogenetics will optimize the pipeline at the stage of research and development of drugs. This method will provide such opportunities as search for new therapeutic targets for drugs through a comprehensive study of the etiopathogenesis of diseases, optogenetic screening, functional optogenetic analysis, and stratification of patients, which is an important step towards personalized medicine [39]. The action mechanisms of the optogenetic component of therapy can encompass regulation of intracellular signaling pathways, increase in permeability of cell membranes, control over cell proliferation and differentiation, upregulation of the active substances of the drug after delivery to the cell, etc.

In addition, optogenetics is considered a promising method for studying the toxicity of drugs at the stage of their development, which is economically feasible: currently used methods are not effective enough – about one third of drugs do not pass clinical trials in phases II-III [40].

Finally, optogenetics can be used as a highly specific tool for drug delivery, allowing to control the speed, rhythm, and dose of the substance released, as well as to overcome histohematic barriers [41].

CONCLUSION

Optogenetics is a promising field of science for solving various biomedical problems. Advances in molecular biology and laser technologies open up new possibilities and allow to solve complex biomedical problems. Improvement and application of this method expand treatment opportunities for a wide range of diseases with minimization of pharmacological effects and considerable efficiency and selectivity of action, which may allow to actively use this method not only in fundamental medicine, but also in practical health care in the future.

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The clinical case of cardiac amyloidosis associated with multiple myeloma

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ABSTRACT

This clinical case demonstrates the difficulty of timely intravital diagnosis of cardiac amyloidosis and the prescription of adequate drug therapy which is associated not only with the limited possibilities of establishing a correct diagnosis and the absence of specific treatment in most cases, but also with a delay in seeking medical care. Thus, development and improvement of non-invasive screening methods of examination will allow to identify this pathology at earlier stages with a possibility of prescribing effective drugs and performing heart transplantation in some cases.

Key words: amyloidosis, multiple myeloma, restrictive cardiomyopathy, endomyocardial biopsy.

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Клинический случай амилоидоза сердца, ассоциированного с миеломной болезнью

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РЕЗЮМЕ

Данный клинический случай демонстрирует сложность своевременной прижизненной диагностики амилоидоза сердца и подбора адекватной медикаментозной терапии, что связано не только с ограниченными возможностями установления точного диагноза и отсутствия в большинстве случаев специфического лечения, но и с поздним обращением пациентов за медицинской помощью. Таким образом, разработка и усовершенствование неинвазивных скрининговых методов обследования позволит выявлять патологию на более ранних этапах с возможностью подбора эффективных препаратов, а в ряде случаев – трансплантации сердца.

Ключевые слова: амилоидоз, миеломная болезнь, рестриктивная кардиомиопатия, эндомиокардиальная биопсия.

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INTRODUCTION

Amyloidosis is a group of diseases with a variety of clinical manifestations characterized by extracelalular deposition of insoluble pathological fibrillar proteins [1]. The pathology was first described by T. Bonet in the 17th century. In the mid-19th century, R. Virkhov applied the term “amyloid”, and in 1937, F.R.B. Atkinson discovered amyloidosis in patients with myeloma [1, 2].

Currently, 4 theories of amyloidogenesis are known: G. Teilum’s theory of local cell genesis, Loeschke-Letterer’s immunological theory, V. Cagli’s theory of dysproteinosis and the mutation theory, but none of them explains the organospecificity and localization of the lesion [3]. The classification of amyloidosis is based on determination of the type of amyloid (A) and precursor protein (A is amyloid A-protein, L is immunoglobulin light chains, TTR is transthyretin, etc.). Clinically, generalized and local forms of the disease are distinguished [1, 3]. The most common type of amyloidosis involving the heart is AL [4]. Idiopathic AL amyloidosis and the one associated with various kinds of monoclonal plasma cell dyscrasias, including multiple myeloma and some other monoclonal gammopathies, are distinguished [5].

Due to late manifestation of the disease, clinical signs are very diverse and may be often disguised as an accompanying pathology (ischemic heart disease, Alzheimer’s disease, renal failure, etc.), which causes late diagnosis and lack of necessary treatment especially in the old age [6, 7]. Common symptoms are the following: hypotonia with syncopal events, chronic heart failure with signs of congestion in both circulations, and heartburn [7]. Currently, along with routine methods of investigation, it is possible to detect amyloidosis more frequently due to introduction of non-invasive screening method, such as speckle tracking echocardiography and magnetic resonance imaging of the heart [8, 9]. Although methods of determining biomarkers of amyloidosis in peripheral blood are being investigated, endomyocardial biopsy followed by histochemical examination is the only method of identifying the type of amyloidosis which allows to timely prescribe adequate drug therapy [10].

The case below is a clinical case of heart amyloidosis associated with myeloma and confirmed by a morphological study.

CLINICAL CASE

Patient S., 67 years old, was hospitalized in the Department of Emergency Cardiology of the Cardiology Research Institute, Tomsk National Research Medical Center in 12.2017 with complaints about shortness of breath of a mixed nature upon exertion, which remits at rest. Thyroidectomy for diffuse toxic goiter in 2007, euthyroidism (L-tyroxine 100 μg). Episodes of non-rhythmic heartbeat and atrial fibrillation were not recorded. Alopecia areata for 3 years. Chronic bronchitis. Loss of body weight by 13 kg in the last 6 months.

In history: 11.2017 she was urgently hospitalized in the district hospital with suspicion of acute coronary syndrome with atypical clinical manifestations and reduction of QRS voltage complexes on ECG. Laboratory diagnosis did not confirm myocardial infarction, but a transthoracic echocardiogram (TTE) revealed hypokinesia of the lower segments. The condition was complicated by pulmonary edema, bilateral hydrothorax, IIB chronic heart failure. Standard drug treatment did not lead to positive dynamics; the patient was transferred to the Cardiology Research Institute. Parameters of blood and urine are presented in Table 1; TTE, ultrasonography of kidneys – in Table 2. Objective clinical examination: BP 90/60 mmHg, hepatomegaly, leg edema. ECG showed sinus tachycardia (HR 104 bpm) and reduction of QRS voltage complex. Myeloma was revealed in laboratory tests. In order to verify the previously diagnosed myocardial infarction, invasive coronary angiography was performed and coronary atherosclerosis was not detected. Given the available restrictive pattern of transmitral blood flow, structural condition of the left ventricle, and minor response to drug treatment, storage disorder was suspected. Magnetic resonance imaging was

carried out, which allowed to visualize both ischemic and non-ischemic (amyloidosis/glycogenosis) damage against the background of myocardial dystrophy (Fig. 2). Endomyocardial biopsy of the right ventricle was performed: PAS-positive substance in the interstitium and endocardium and amyloid deposits were determined.

On the basis of all the data, it was possible to verify the diagnosis of secondary amyloidosis of the heart, probably AL type, associated with myeloma. Against the background of therapy with beta-blocker, inhibitor of ACE, and diuretics, hydrothorax was relieved; however, persistent hypotonia, pronounced fatigue, insomnia, and decreased appetite were preserved. The patient was transferred to the Department of Nephrology and Chronic Hemodialysis, where bone marrow trepanobiopsy was performed and myeloma and kidney amyloidosis were confirmed. Hydrothorax and hydropericardium in the intensive care unit recurred, fatigue increased, cachexia, hypotonia, and pulmonary edema recurred. The patient died on 8.01.2018.

This clinical case demonstrates the difficulty of timely intravital diagnosis of amyloidosis and selection of adequate drug therapy, which is associated not only with limited possibilities of establishing an accurate diagnosis and absence of specific treatment in most cases, but also with a delay in seeking medical care. Thus, the development and improvement of non-invasive screening methods will allow to detect the pathology at earlier stages with the possibility to select effective drugs and perform heart transplantation in some cases.

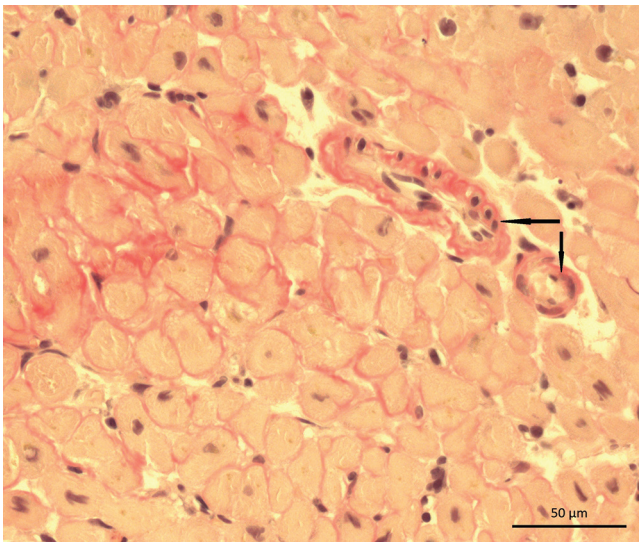

Figure. Myocardial biopsy

Table 1.

Laboratory parameters		
Blood test		
	12.12.2017	Normal
White blood cells, 10^9/L	6.8	4.0–9.0
Red blood cells, 10^{12}/L	5.03	3.9–4.7
Hemoglobin, g/L	163	120–140
ESR, mm/h	6	2–20
Blood biochemistry		
CPK-MB, U/L	22	0–25
Creatinine, µmol/L	72	53–97
Urea, mmol/L	5.9	2.2–7.2
Cholesterol, mmol/L	5.9	3.5–5.2
Total protein, g/L	49	64–83
CRP, mg/L	4.0	0–10.0
Potassium, mmol/L	3.8	3.5–5.1
Urinalysis		
White blood cells	10–12	0–3
Protein, g/L	5	0–0.08
Bence – Jones protein	+++	
Daily protein excretion, g/day	4.98	0–0.14

Table 2

Instrumental parameters		
Echocardiography 12.12.2017		
		Normal
Left atrium, mm	50x64	43 × 49
Right atrium, mm	47x61	43 × 49
LAV, ml	100.8	20–59
RAV, ml	97	19–64
LVED, ml	41	50–112
LVES, ml	18	12–41
RVED, mm	33	36–51
RVES, mm	21	21–34
EF LV (B), %	60	55–78
SV LF, ml	27	39–74
CI, L/min/m^2	1.9	1.7–4.5
Interventricular septum, mm	16	6.4–9.2
LV posterior wall, mm	16	6.4–9.2
Myocardial mass, g	200	< 146
Myocardial mass index, g/m^2	136	44–100
RVSP, mmHg	52	20–32
Vena cava inferior, mm	23	< 21
E/A	2	0.62–1.39
E/ e`	21	< 8

Ultrasound of the kidneys 18.12.2017			
	Right	Left	Normal
Length, mm	107	101	90–120
Width, mm	60	52	45–60
Parenchymal thickness, mm	13.7	12	12–20
Cyst	12 mm	–	–

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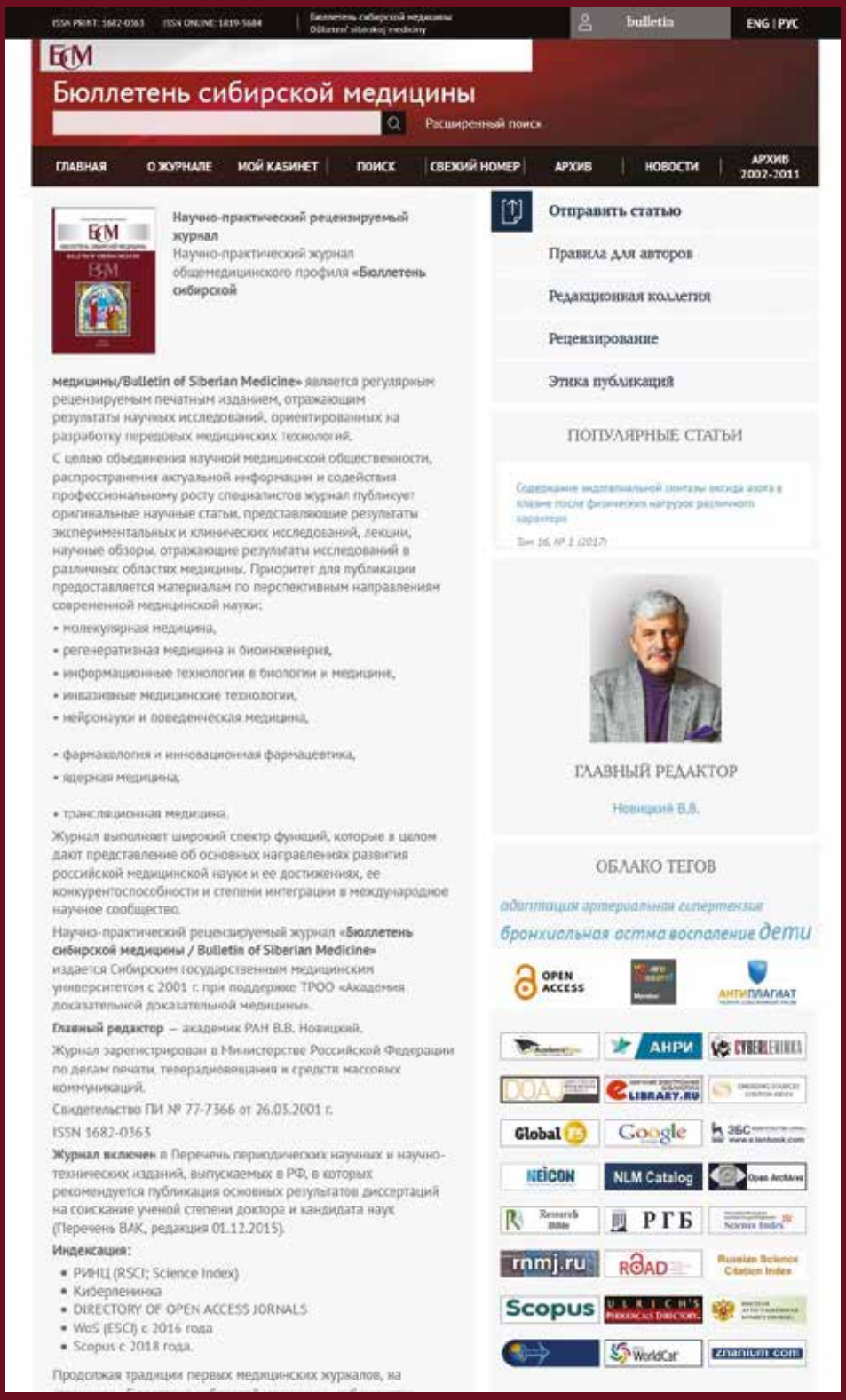

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