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Features of vasoactive substance regulation in chorionic villi in women with spontaneous abortion and active cytomegalovirus infection

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ABSTRACT

The aim of the study was to assess the levels of soluble *fms*-like tyrosine kinase 1 (sFlt1), placental growth factor (PlGF), and vascular endothelial growth factor A (VEGF-A) in tissue extracts in comparison with the histologic examination of the endometrium and chorionic villi in women with spontaneous abortion and active cytomegalovirus (CMV) infection.

Materials and methods. 81 women at 7–9 weeks of pregnancy were examined: of them, 51 women were CMV-seropositive with active infection and after spontaneous abortion, and 30 patients were CMV-seronegative, healthy women after therapeutic abortion. Immunoglobulins (Ig) M and G to CMV and CMV IgG avidity were measured in the blood plasma; sFlt1, PlGF, and VEGF-A were determined in extracts of chorionic villi by enzyme immunoassay. CMV DNA was detected in mononuclear cells of peripheral blood, urine, and chorionic villi by real-time polymerase chain reaction (PCR). A histologic examination of the endometrium and chorionic villi was carried out.

Results. In chorionic villus extracts of women with spontaneous abortion and active CMV infection, the concentration of sFlt1 was 3.25 times higher ($p < 0.001$), and the levels of PlGF and VEGF-A were 1.31 ($p < 0.001$) and 2.16 times lower ($p < 0.001$) than in healthy women. A strong negative correlation was established between the levels of sFlt1 and PlGF ($r = –0.702$; $p < 0.001$) and VEGF-A ($r = –0.858$; $p < 0.0005$), and a positive correlation was revealed between PlGF and VEGF-A levels ($r = 0.860$; $p < 0.001$). According to the data of the histologic examination, a lag in decidual transformation of uterine vessels, trophoblast invasion, growth and differentiation of villi, and formation of fetal circulation was detected.

Conclusion. The mechanisms of spontaneous abortion in women with active CMV infection include an imbalance of pro- and anti-angiogenic factors, which causes impaired placental development and uteroplacental circulation.

Keywords: pregnancy, spontaneous abortion, cytomegalovirus infection, chorionic villi, anti-angiogenic factors, vasoactive substances

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Особенности вазоактивной регуляции в ворсинчатом хорионе у женщин с самопроизвольным абортом и активной цитомегаловирусной инфекцией

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РЕЗЮМЕ

Цель. Оценить содержание растворимого рецептора *fms*-подобной тирозинкиназы (sFlt1), плацентарного фактора роста (PlGF) и васкулоэндотелиального фактора роста (VEGF-A) в тканевых экстрактах в сопоставлении с гистологией слизистой оболочки матки и ворсинчатого хориона у женщин с самопроизвольным абортом и активной цитомегаловирусной (ЦМВ) инфекцией.

Материалы и методы. Обследована 81 женщина в период с 7-й по 9-ю нед беременности: 51 ЦМВ-серопозитивная с самопроизвольным абортом и активной инфекцией и 30 серонегативных здоровых женщин с медицинским абортом. В плазме крови определяли иммуноглобулины (Ig) класса M и G к ЦМВ, авидность ЦМВ-IgG; в экстрактах ворсинчатого хориона – sFlt1, PlGF, VEGF-A методом иммуноферментного анализа. В мононуклеарных клетках крови, пробах мочи, ворсинчатом хорионе методом полимеразной цепной реакции в режиме реального времени выявляли ДНК ЦМВ. Проводили гистологическое исследование слизистой оболочки матки и ворсинчатого хориона.

Результаты. В экстрактах ворсинчатого хориона у женщин с самопроизвольным абортом и активной ЦМВ-инфекцией концентрация sFlt-1 была выше в 3,25 раза ($p < 0,001$), PlGF и VEGF-A – ниже в 1,31 ($p < 0,001$) и 2,16 раза ($p < 0,001$), чем у здоровых женщин. Установлена сильная обратная корреляционная связь между уровнями sFlt-1 и PlGF ($r = –0,702$; $p < 0,001$) и VEGF-A ($r = –0,858$; $p < 0,0005$), прямая связь – PlGF и VEGF-A ($r = 0,860$; $p < 0,001$). По данным гистологического исследования, отмечено отставание в децидуализации и трансформации маточных сосудов, инвазии трофобласта, роста и дифференцировки ворсин, формирования фетальных сосудов.

Заключение. К механизмам самопроизвольного аборта у женщин с активной ЦМВ-инфекцией можно отнести дисбаланс анти- и проангиогенной регуляции, вызывающий ограничение развития плаценты и маточно-плацентарного кровообращения.

Ключевые слова: беременность, самопроизвольный аборт, цитомегаловирусная инфекция, ворсинчатый хорион, антиангиогенные факторы, вазоактивные факторы

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

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INTRODUCTION

A number of adverse pregnancy outcomes caused by impaired placental formation and associated processes of invasion and uteroplacental angiogenesis [1–3] occur regardless of whether or not the embryo and placenta are infected with cytomegalovirus (CMV) [4]. The mechanism of disturbances in the placental morphogenesis in the presence of CMV infection reactivation is based on a systemic inflammatory response that causes changes in cytokine profiles in the placenta [5], inducing local inflammation [6], and apoptosis in uninfected trophoblasts [7] and vascular smooth muscle cells surrounding the spiral arteries of the decidua [8], which leads to hypoxia.

Placental growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family involved in the neoangiogenesis. Its secretion is determined by partial pressure of oxygen and alters during pregnancy. PlGF binds the VEGF receptor (Flt1) and receptor tyrosine kinase, which leads to stimulation of pro-angiogenic factor formation that regulate proliferation and differentiation of mesenchymal cells in the villous stroma into endothelial cells [9]. Its soluble splice variant sFlt1 binds free PlGF and, therefore, counteracts its effects [10]. Serum levels of sFlt1 and PlGF, especially their ratio, are commonly used to predict the risk of preeclampsia in women [11], including CMV etiology [12]. At the same time, studies on the concentration of sFlt1, PlGF, and VEGF-A in the chorionic villi in women with spontaneous abortion and active CMV infection have not been carried out. There is also no histologic description of the chorionic villi and vessels in the early placental villi in this pathology in women.

The aim of the study was to assess the level of sFlt1, PlGF, and VEGF-A in tissue extracts compared with the histologic examination of the endometrium and chorionic villi in women with spontaneous abortion and active CMV infection

MATERIALS AND METHODS

The study was carried out in accordance with the principles set out in the Declaration of Helsinki and approved by the Biomedical Ethics Committee at the Far Eastern Scientific Center of Physiology and Pathology of Respiration (Blagoveshchensk) (protocol No. 15 of 25.02.2017). All women included in the study gave a written informed consent to collection of samples and subsequent analysis.

Groups of women for the study were formed at the gynecology department of City Clinical Hospital (Blagoveshchensk) from 2017 to 2019. 81 women at 7–9 weeks of pregnancy were examined: of them, 51 women were CMV-seropositive with active infection and after spontaneous abortion (treatment group), and 30 patients were CMV-seronegative, healthy women after therapeutic abortion (control group). Active infection was determined by the presence of detectable type-specific antibodies to CMV (immunoglobulins (Ig) M,) IgG avidity of more than 50%, and CMV DNA in blood or urine. Exclusion criteria were the presence of any immunodeficiency, endocrine disorders, and primary CMV infection. All women were comparable in age (treatment group – 26.7 ± 3.17, control group – 27.3 ± 3.29, $p > 0.05$), body mass index (treatment group – 23.01 ± 1.09, control group – 22.93 ± 1.28, $p > 0.05$), and social status.

Blood samples from women were collected by venipuncture into tubes containing sodium citrate upon admission to the gynecology department. Blood plasma was obtained by centrifugation for 20 min at 3,000 revolutions per minute. Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll – urografin density gradient centrifugation (ρ = 1.077 g / cm^3) in accordance with the manufacturer's instructions (DNA – Technology, Russian Federation). Urine samples were obtained by centrifugation for 10 min at 13,000 revolutions per minute, followed by resuspension of the sediment in a sterile environment. Biological samples (endometrium, chorionic villi) were collected within 10–15 minutes after therapeutic abortion and washed in sterile saline before use. To isolate DNA, pre-weighed pieces of chorionic villi were placed into liquid nitrogen and ground with a pestle in liquid nitrogen; an equal volume of sterile saline was added, the mixture was thoroughly mixed, and the required volume of the material was selected for further analysis. The tissue extract was obtained by centrifuging the homogenate produced by the method described above for 15 minutes at 13,000 revolutions per minute and a temperature of +4° C.

All biological samples (plasma, PBMCs, urine, extracts of chorionic villi) were frozen and stored at -70° C until the analysis.

IgM and IgG to CMV were detected in paired plasma samples using VectoCMV-IgM and VectoCMV-IgG ELISA reagent kits (Russian Federation) on the Stat Fax 2100 reader (USA). To assess serotype-specific antibodies, the cutoff index (CI) was calculated. The result of the analysis was considered positive if the CI was ≥ 1.1. Analysis of CMV IgG avidity was performed in blood using standard VectoCMV

IgG avidity ELISA kits (Russian Federation). Avidity index (AI) of > 50% indicated the presence of highly avid IgG antibodies and chronic infection. Quantitative determination of VEGF-A and VEGFR1 / sFlt1 (Bender MedSystems, USA), as well as PlGF (Quantikine ELISA Kit, USA) was carried out in extracts of chorionic villi using enzyme-linked immunosorbent assay (ELISA). All ELISA tests were performed in strict accordance with the manufacturer's instructions for commercial reagent kits.

Extraction of DNA in the biological material (PBMCs, urine samples, extracts of chorionic villi) was performed using the Proba-Rapid reagent kit (DNA – Technology, Russian Federation) in accordance with the manufacturer's instructions.

Detection of CMV DNA was carried out using the CMV-GEN reagent kit (DNA – Technology, Russian Federation) intended for amplification of DNA using real-time polymerase chain reaction (PCR). Quantitative detection of PCR amplification products was carried out using a DT-96 device (DNA – Technology, Russian Federation), according to the manufacturer's recommendations.

For histologic studies, pieces of chorionic villi were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer with subsequent additional fixation in 1% osmium tetroxide solution. Semithin sections were obtained on the LKB NOWA 8800 ultramicrotome (Sweden), stained with toluidine blue, and viewed and documented using the Meiji digital camera (Austria).

Statistical analysis and processing of the data were performed using the IBM SPSS Statistics 23.0 software package (USA). All results were checked for normality using the Shapiro – Wilk test. Quantitative data were presented as $M \pm SD$, where M is the arithmetic mean, and SD is the standard deviation. Due to a lack of normal distribution in the groups, the nonparametric Mann – Whitney test was used to describe the statistical differences. The differences were considered statistically significant at $p < 0.05$. To identify a relationship between the studied parameters, the Spearman's rank correlation coefficient (r) was calculated.

RESULTS

All women in the treatment group admitted to the gynecology department of the City Clinical Hospital (Blagoveshchensk) with clinical signs of spontaneous abortion (lower abdominal pain, bloody vaginal discharge) at 7–9 weeks of gestation were diagnosed with chronic CMV at the stage of active infection. The level of IgM antibodies to CMV, assessed by the CI, was 3.71 ± 0.46, the level of IgG antibodies was 18.83 ± 2.25, and IgG avidity was 86.83 ± 2.20 %. Urine was positive for CMV DNA in 24 women (47.06%). All samples of chorionic villi obtained from CMV-seropositive women with active infection following instrumental revision of the uterine cavity were negative for CMV DNA.

The results of ELISA tests of the extracts of chorionic villi obtained from women with spontaneous abortion and active CMV infection (treatment group) and healthy CMV-seronegative women (control group) are presented in the Table. It was shown that the content of the antiangiogenic factor sFlt1 in the extracts of chorionic villi in the treatment group was 3.3 times higher than in the control group. The levels of vasoactive factors PlGF and VEGF-A in the treatment group were significantly lower (by 1.3 times and 2.2 times, respectively) compared with the same parameters in the control group.

Table

The content of sFlt1, PlGF, and VEGF-A in the extracts of chorionic villi in the studied groups, $M \pm SD$

Parameter	Group	
	treatment	control
Number of studies	51	30
sFlt1, pg / ml	12.39 ± 0.30*	3.74 ± 1.25
PlGF, pg /ml	55.26 ± 0.41*	73.01 ± 1.29
VEGF-A, pg / ml	17.22 ± 0.50*	37.65 ± 1.52

Note: sFlt1 – soluble receptor of *fms*-like tyrosine kinase 1, PlGF – placental growth factor, VEGF-A – vascular endothelial growth factor A.

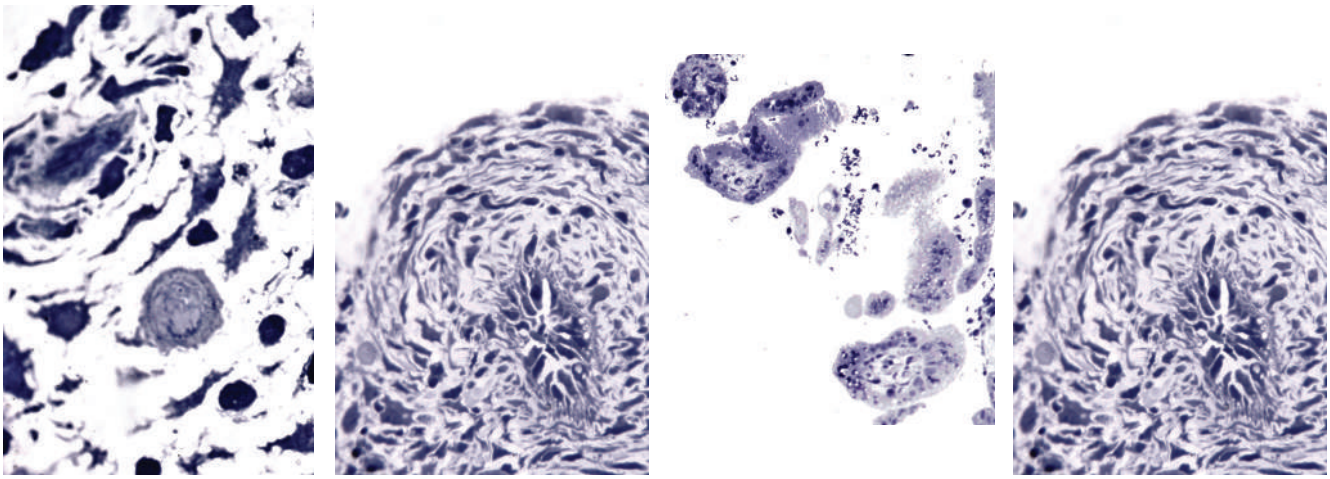
* – statistical significance of differences between the parameters compared with the control group ($p < 0.001$).

The analysis of correlations between the studied parameters in the treatment group showed the presence of a strong inverse relationship between sFlt1 and PlGF ($r = –0.702$, $p < 0.001$) and VEGF-A ($r = –0.858$, $p < 0.001$) and a direct relationship between PlGF and VEGF-A ($r = 0.860$, $p < 0.001$). In the control group, a strong direct relationship was revealed between PlGF and VEGF-A ($r = 0.958$, $p < 0.001$).

At the next stage of the study, a histologic analysis of microsections of the endometrium and chorionic villi obtained from women with spontaneous abortion and active CMV infection was performed. The microsections of the endometrium revealed focal stromal edema with zones of inflammatory infiltrate (Figure, *a*). Decidualization of the stroma was slowed down. Interstitial cytotrophoblast invasion was detected

in 53% of cases only in the superficial parts of the functional layer (Figure, b). Invasive cytotrophoblast located in the subendothelial layer was detected in 44.5% of cases. The spiral arteries in the endometrium in most cases were narrowed, endothelial cells underwent dystrophic changes, which indicated insufficient angiogenic factor stimulation of uterine spiral artery remodeling.

On the microsections of the chorionic villi, mesenchymal villi without signs of differentiation into endothelial cells predominated. Necrotic avascular villi were observed (Figure, *c*). Intermediate hypovascular villi showed signs of apoptosis of endothelial cells and fibroblasts (Figure, *d*). Fetal capillaries were stretched and compressed as a result of villous edema. The epithelium of these villi had uneven thickness. Areas with thickenings and chaotically located, deformed nuclei, as well as with delayed cytotrophoblast formation were often observed. These data indicate impaired formation of chorionic villi and invasion / migration of the trophoblast, which determines further fetal development under conditions of active CMV infection.



a *b* *c* *d*

Figure. Lining of the uterus (*a, b*) and chorionic villi (*c, d*) in women with spontaneous abortion and active CMV infection. Semithin sections. Staining with toluidine blue. *a, b, d* – ×400, *c* – ×200

DISCUSSION

Impaired mechanisms of vasoactive substance regulation in the formation of chorionic villi and uteroplacental vessels, observed in women under the conditions of active CMV infection, results in insufficient oxygen supply and impaired trophism and excretion of products of placental metabolism through the maternal bloodstream [13]. Chronic oxygen deficiency and high levels of prooxidants and proinflammatory factors, shown in our earlier works [14, 15] and in the studies by other authors [16], may contribute to a change in placental perfusion, causing inactivation of the Ca^{2+}-dependent SK3 channel (regulator of the vascular tone) and detachment of the soluble splice variant from the Flt1 transmembrane domain [17]. As a result, high levels of sFlt1 entering the maternal bloodstream and free circulating in it under the conditions of active CMV infection limit the expression and bioavailability of vasoactive factors PlGF and VEGF-A by the trophoblast and vascular endothelium [18], which leads to retarded growth and differentiation of villi, invasion / migration of the trophoblast, and limitation of uteroplacental angiogenesis.

It should also be noted that high levels of sFlt1, like reactive oxygen species and proinflammatory cytokines, inhibit the soluble guanylate cyclase (sGC) / cyclic guanosine monophosphate (cGMP) signaling pathway, thereby causing uterine artery vasoconstriction and blood flow to the embryo [19], which was confirmed by histologic studies. Degradation and decreased signaling activity of cGMP may also indicate suppression of proliferation and differentiation [19], which was manifested through a decrease in interstitial trophoblast invasion in women with active CMV infection.

Consequently, the established imbalance of vasoactive substances in the chorionic villi, evidenced by a strong inverse relationship between sFlt1 and PlGF and VEGF-A, may cause spontaneous abortion in the absence of CMV infection in the placental tissues. A number of other studies showed a relationship of high levels of sFlt1 with development of endothelial dys-

function, which causes severe complications of pregnancy, such as preeclampsia and fetal growth restriction [20, 21], as well as with infertility and recurrent pregnancy loss [22].

Based on the obtained results of the study, it can be concluded that pathology of placental development and function, which determines vessel regression, and insufficient blood supply due to imbalance of vasoactive substances are the main causes and mechanisms of impaired development and death of the embryo under the conditions of active CMV infection.

CONCLUSION

We have shown that extracts of the chorionic villi obtained from women with spontaneous abortion and active CMV infection are characterized by increased levels of sFlt-1 and low concentrations of PlGF and VEGF-A. The presence of a strong inverse correlation between anti- and proangiogenic factors indicates some tension in the system regulating the development of the placenta and uteroplacental vessels, which was confirmed by histologic studies. The resulting angiogenic changes under the conditions of active CMV infection reduce the increase in the uteroplacental circulation, which restricts the growth and development of the embryo, contributing to its death.

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Causal relationship between allergy and seborrheic dermatitis

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ABSTRACT

Seborrheic dermatitis is a chronic relapsing inflammatory skin disease associated with overproduction of sebum and activation of the fungal skin microbiota characterized by the presence of erythematous pruritic patches and plaques with greasy scales in areas rich in sebaceous glands.

Aim. To study the spectrum of sensitization to food, pollen, and indoor and fungal allergens in patients with seborrheic dermatitis.

Materials and methods. The study researched features of the spectrum of sensitization to food, pollen, and fungal and indoor allergens in patients with seborrheic dermatitis (n = 40, aged 15–59 years) based on the data of an objective examination and the results of an allergen-specific test, including skin prick testing.

Results. It was determined that the most significant food allergens in seborrheic dermatitis are chicken eggs and grains. The incidence of polyvalent sensitization to food allergens was 40.0%. Additionally, high incidence of sensitization to pollen allergens, most often to weed and poaceae pollen, was revealed in patients with seborrheic dermatitis. Among indoor allergens, the highest incidence of sensitization was determined to house dust and *Dermatophagoides pteronyssinus*. Among fungal allergens, the highest incidence of sensitization was detected to *Candida albicans*.

Conclusion. It was found that patients with seborrheic dermatitis are often sensitized to food, pollen, and indoor and fungal allergens. Therefore, allergy can be considered a risk factor for the development of pathology.

Keywords: seborrheic dermatitis, allergens, sensitization, *Candida albicans, Dermatophagoides pteronyssinus, Malassezia*

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at the Research Institute of Medical Problems of the North (Protocol No. 12 of 10.12.2013).

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Аллергия и себорейный дерматит – причинно-следственная взаимосвязь

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РЕЗЮМЕ

Себорейный дерматит – хроническое рецидивирующее воспалительное заболевание кожи, связанное с гиперпродукцией кожного сала и активацией грибковой микрофлоры кожи, характеризующееся наличием эритематозных зудящих пятен и бляшек с жирными чешуйками в зонах с большим скоплением сальных желез.

Цель – изучить спектр сенсибилизации больных себорейным дерматитом к пищевым, пыльцевым, бытовым и грибковым аллергенам.

Материалы и методы. Изучены особенности спектра сенсибилизации больных себорейным дерматитом (n = 40, возраст 15–59 лет) к пищевым, пыльцевым, грибковым и бытовым аллергенам на основании данных объективного осмотра и результатов специфического аллергологического обследования, включая кожное тестирование (prick-тест).

Результаты. Определено, что наиболее значимыми пищевыми аллергенами при себорейном дерматите являются куриное яйцо и пищевые злаки. Частота встречаемости поливалентной сенсибилизации к пищевым аллергенам обнаружена в 40% случаев. Также определена высокая частота сенсибилизации больных себорейным дерматитом к пыльцевым аллергенам, чаще всего к аллергенам пыльцы сорных и злаковых трав. Среди бытовых аллергенов наиболее высокая частота встречаемости сенсибилизации определена к домашней пыли и *Dermatophagoides pteronyssinus*, среди грибковых аллергенов – к *Candida albicans*.

Заключение. Установлено, что больные себорейным дерматитом нередко сенсибилизированы к пищевым, пыльцевым, бытовым и грибковым аллергенам, и, следовательно, аллергию можно рассматривать как фактор риска развития патологии.

Ключевые слова: себорейный дерматит, аллергены, сенсибилизация, *Candida albicans, Dermatophagoides pteronyssinus, Malassezia*

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

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

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом НИИ МПС (протокол № 12 от 10.12.2013).

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INTRODUCTION

Seborrheic dermatitis (SD) is a chronic relapsing inflammatory skin disease associated with overproduction of sebum and activation of fungal skin microbiota, characterized by the presence of erythematous pruritic patches and plaques with greasy scales in areas rich in sebaceous glands on the scalp, face (forehead, nasolabial triangle), trunk, and intertriginous areas [1–3]. In severe cases of scalp lesions in patients with SD, thinning and loss of hair with the formation of alopecia are observed [4, 5]. A steady increase in the incidence of SD with a growing number of severe forms, torpidity to local treatment, and a negative impact on the quality of life in patients determine the relevance of studying this problem.

The etiology and pathogenesis of SD are not fully understood. It is believed that the development of SD is promoted by the activation of the lipophilic yeasts *Malassezia spp.*, increased secretion of sebum, and a change in its qualitative composition against the background of psychoemotional overstrain, stressful situations, hormonal, immune and neuroendocrine disorders, and taking certain medications [4]. A recent study found that in patients with SD, high colonization of *Staphylococcus epidermidis* is determined in comparison with the controls [6]. The literature describes an increase in the incidence of SD in patients with HIV and Parkinson's disease, which indicates the presence of an immune imbalance in this pathology [4, 5].

Therefore, SD is a multifactorial disease, the pathogenesis of which involves immune and environmental factors. The role of nutrition in the development of SD is undeniable. There is an opinion that skin diseases are often associated with changes in the intestinal microbiota accompanied by the impaired mucosal barrier [3, 7]. Food allergens can easily penetrate through the damaged intestinal mucosal barrier and trigger skin rashes [3, 8]. In addition, damage to the epidermal barrier in SD can contribute to the percutaneous penetration of aeroallergens and sensitization of patients. At the same time, data on the spectrum of sensitization to various groups of allergens in SD are extremely scarce, which determines the relevance of the study.

The aim of the study was to analyze the spectrum of sensitization to food, pollen, and indoor and fungal allergens in patients with SD.

MATERIALS AND METHODS

The study included patients with SD (n = 40) aged 15–59 years. The average age of patients was 31.6 ± 1.5 years. The average duration of the disease was 6.2 ± 0.9 years. The average age of the onset of SD was 25.3 ± 1.7 years. The diagnosis of SD was based on the presence of specific clinical signs: rashes on the skin of the scalp, face, and trunk (in the chest and interscapular region), represented by round, oval or irregular-shaped foci of hyperemia with fuzzy boundaries and grayish-yellow scales on the surface. A specific allergological examination (allergological history, skin testing) was carried out to diagnose allergy.

The study of the sensitization spectrum to food, pollen, and indoor and fungal allergens was carried out by skin prick testing, taking into account the size of the blister and the magnitude of hyperemia: weakly positive reaction – 3–5 mm (+), positive – 6–9 mm (++), strongly positive – 10–14 mm (+++), hyperergic – 15 mm and more (++++). We used the following allergens (Allergopharma, Germany): food allergens – cow's milk (cow's milk protein, casein), beef, chicken egg (protein, yolk, whole egg), chicken meet, food grains (wheat and rye flour, barley, and oatmeal); pollen allergens – mixtures of tree pollen (birch, oak, maple, hazel, alder), cereals (prickly thrift, bonfire, foxtail, bluegrass, fescue, wheat grass, ryegrass, rye), and weed grass (orach, wormwood, sunflower); indoor allergens – *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, house dust; fungal allergens – *Candida albicans*, *Cladosporium herbarum*, *Penicillium notatum*.

Statistica 6.0 software package (StatSoft Inc., USA) was used for statistical analysis. Statistical processing of the results was carried out with the calculation of generalized coefficients: the mean value (M) and the error of the mean (m). When analyzing qualitative characteristics, the relative frequency of the feature (prevalence) p was assessed, and the average error for the sample fraction m was determined [9]. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The main clinical manifestations of seborrheic SD are erythematous foci covered with yellowish scales and crusts. Pruritus was observed in 82% of patients (n = 33). 42% of patients (n = 17) had manifestations of SD on the facial skin, and 7% of patients (n = 3) had SD manifestations on the skin of the trunk.

In 75% of cases (n = 30), patients had an aggravated allergy history (drug allergy, atopic dermatitis, allergic rhinitis, and urticaria). Thus, manifestations of perennial allergic rhinitis were identified in 50% of patients (n = 20), and signs of atopic dermatitis were detected in 35% of patients (n = 14). An aggravated family history

of allergic diseases in patients with SD was detected in 40% of cases (n = 16). Aggravated heredity for SD was noted in 15% of cases (n = 6).

The analysis of the results of skin testing revealed hypersensitivity to food allergens in 95% of patients (n = 38); sensitization was strongly positive in 42% of cases and positive in 39% of cases. It was determined that the most significant food allergens in SD patients were chicken eggs and grains, sensitization to which was determined in 74.3% and 75.0% of cases, respectively (Table).

Table

Features of the spectrum of sensitization to food, pollen, indoor, and fungal allergens in patients with seborrheic dermatitis

Allergen	Number of examined patients, (n / N)	Prevalence of sensitization, %, $M \pm m$
Food allergens		
Cow's milk	25/40	62.5 ± 7.6
Beef	17/35	48.6 ± 8.4
Chicken egg	29/39	74.3 ± 6.9
Chicken meat	15/37	40.5 ± 8.1
Grains	30/40	75.0 ± 6.8
Pollen allergens		
Meadow grass	20/35	57.1 ± 8.4
Trees	19/34	55.9 ± 8.5
Weed grass	21/35	60.0 ± 8.3
Poaceae	21/35	60.0 ± 8.3
Indoor allergens		
Dermatophagoides pteronyssinus	13/20	65.0 ± 10.9
Dermatophagoides farinae	11/21	52.4 ± 11.2
House dust	16/21	76.2 ± 9.5
Fungal allergens		
Candida albicans	18/39	46.2 ± 8.0
Cladosporium herbarum	12/29	41.4 ± 9.3
Penicillium notatum	8/29	27.6 ± 8.4

Note: N – the number of tested patients, n (%) – absolute (relative) number of sensitized patients.

According to the literature, food allergy to chicken eggs is one of the most common in the world, therefore, high sensitization to this allergen in patients with SD was expected [10]. To a lesser extent, sensitization to cow's milk was widely determined in 62.5% of cases. According to the literature, cow's milk is one of the main causes of food allergy in the first year of life, however, it is rare in adults. Meanwhile, it is known that the presence of food allergy to cow's milk in adults is manifested by severe clinical forms of the disease [11].

The literature data on the incidence of monovalent and polyvalent sensitization (to 3 or more allergens) to food allergens are different [10]. The incidence of polyvalent sensitization to food allergens in our study was 40% (n = 16 / 40), bivalent sensitization was revealed in 37% of cases (n = 15 / 40), monovalent sensitization – in 17% of cases (n = 7 / 40).

According to the results of skin testing, all patients were prescribed an elimination diet in combination with standard topical therapy. In 85% of cases, patients noted a significant improvement in skin processes by the 3rd week of treatment: reduction of itching, hyperemia, and peeling in the foci of SD.

High incidence of sensitization to pollen allergens, most often to weed and poaceae pollen, was determined in patients with SD (Table). Classical manifestations of hay fever in the form of seasonal allergic rhinoconjunctivitis in patients with SD were noted in 12% of cases (n = 5 / 40). High incidence of sensitization to pollen allergens in patients with SD can be due to common antigenic determinants with food allergens and, as a consequence, development of cross-reactivity.

Skin testing with indoor allergens showed the highest incidence of sensitization to house dust and *Dermatophagoides pteronyssinus* in patients with SD: 76.2% and 65.0%, respectively (Table). It is known that the development of SD is associated with the activation of fungal skin microbiota, which may result in the development of fungal sensitization [3]. As a result of skin testing with fungal allergens, the highest incidence of sensitization in patients with SD was noted to *Candida albicans* (46.2% of cases (n = 18 / 39)) (Table).

CONCLUSION

The study showed high incidence of sensitization to food, pollen, indoor, and fungal allergens in patients with SD. The most significant food allergens in SD were chicken eggs and grains. Despite the fact that the incidence of food allergy among adults is only about 2% [10, 12], the defined spectrum of sensitization to food allergens and the positive effect of the elimination diet in patients with SD proves the important role of food allergens as triggers of the disease. According to experts, every year there is an increase in the incidence of food allergy in the world, which is associated with a change in the nature of nutrition in the population of various countries and the emergence of new food processing technologies [12].

Sensitization to pollen allergens in patients with SD can be the result of common antigenic determinants with food allergens. It is known that patients with hay fever have cross-reactivity to fruits and vegetables, as the result of the presence of homologous proteins with

plant pollen. Given high incidence of pollen sensitization in patients with SD, it can be assumed that in some cases, eating foods that have common antigenic determinants with pollen allergens can result in expanding the spectrum of sensitization and aggravating pathology as a result of cross-reactivity.

In turn, impaired skin barrier function in patients with SD can contribute to the penetration of various aeroallergens into the body. High incidence of sensitization to house dust and *Dermatophagoides pteronyssinus* in patients with SD deserves special attention. Recently, the literature dedicated to skin microbiota in SD patients has presented data on colonization of lesions by *Staphylococcus epidermidis* and *Staphylococcus aureus* [6, 13]. House dust mites can serve as carriers of bacteria of the Staphylococcaceae family, responsible for the induction of IgE-mediated sensitization to microbial antigens [14]. Therefore, high incidence of sensitization to *Dermatophagoides pteronyssinus* in patients with SD, which we identified, can be not only one of the important etiological factors of the pathology, but also induce an infectious allergy.

Sensitization to *Candida albicans* in patients with SD is of great interest. Yeast-like fungi of the genus *Candida*, being skin commensals, play a certain role in the pathogenesis of allergic diseases, such as atopic dermatitis [15]. The presence of sensitization to fungal allergens in the patients can be associated with cross-reactivity between *Candida albicans* and fungi of the genus *Malassezia*, whose role in the pathogenesis of SD is actively discussed [2, 3]. It is believed that SD arises following an immune response to fungal antigens of the genus *Malassezia* and products of their metabolism [2]. The pathological immune response contributes to increased penetration of *Malassezia* and *Candida albicans* through the epidermis, which leads to sensitization of the body and a continuous cycle of inflammation.

Despite the interesting data we obtained on the presence of a wide spectrum of sensitization to allergens of various origin in patients with SD, further in-depth specific allergological studies are required, including other diagnostic (elimination and provocation tests) and therapeutic (elimination diets, allergen-specific immunotherapy) measures in order to confirm the role of allergy as a trigger of the pathology. In addition, it can be assumed that SD may be one of the atypical clinical manifestations of allergy.

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Pathogenetic features of experimental osteoarthrosis induced by dexamethasone and talc

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Abstract

The aim of the study was to investigate the pathogenesis of experimental osteoarthrosis induced by dexamethasone and talc by examining the structure and defining the morphometric and metabolic features of knee joint skeletal connective tissues in rats.

Materials and methods. We performed a morphometric evaluation of articular cartilages (their thickness, extracellular matrix arrangement, spatial arrangement of the main components, distribution density, and main cellular indices of chondrocytes), as well as changes in subchondral bones (the presence of trabeculae in the basal layer of the articular cartilage and individual osteophytes) in 30 rats with a model of primary osteoarthrosis induced by sequential administration of 0.5 ml dexamethasone (2 mg) and 1 ml 10% sterile talc suspension mixed with normal saline into the joint cavity. We studied the histologic specimens of the knee joints stained with hematoxylin – eosin, Alcian blue (pH 1.0 and 2.5), as well as with Van Gieson's, Masson's, and Mallory's trichome stains. The metabolic features of the articular cartilage and bone tissues were investigated by determining the hyaluronan, osteocalcin, and type I collagen levels in the serum of the rats.

Results. In the rats with dexamethasone- and talc-induced osteoarthrosis, the thickness of cartilages in their weight-bearing areas decreased by 50%, the spatial arrangement of chondrocytes was impaired, and the nuclear – cytoplasmatic ratio ($p < 0.01$) decreased to 0.3. Besides, a rise in the serum levels of hyaluronan ($p < 0.001$) to 110.2 ng / ml, type I collagen fragments ($p < 0.001$) to 217.9 ng / ml, and osteocalcin ($p < 0.001$) to 231.1 ng / ml was detected.

Conclusion. The main pathogenetic features of experimental osteoarthrosis induced by dexamethasone and talc include impaired distribution density, morphological characteristics, and functional activity of chondrocytes, which results in inhibited synthesis of extracellular matrix components in the articular cartilage and activated destruction of proteoglycans containing unsulphated glycosaminoglycans. The subchondral bone remodeling in experimental osteoarthrosis induced by dexamethasone and talc is characterized by intensification of synthetic activity of osteoblasts.

Keywords: osteoarthrosis, rats, dexamethasone, articular cartilage, chondrocytes, osteocalcin, subchondral bone, hyaluronic acid, type I collagen

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Conformity with the principles of ethics. The study was approved by the local Ethics Committee at Saratov State Medical University named after V.I. Razumovsky (Protocol No. 2 of 02.10.2018).

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Патогенетические особенности экспериментального остеоартроза, индуцированного дексаметазоном и тальком

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РЕЗЮМЕ

Цель – изучение патогенеза экспериментального остеоартроза (ОА), индуцированного дексаметазоном и тальком, на основании исследования структуры, определения морфометрических характеристик и метаболических особенностей скелетных соединительных тканей коленных суставов у крыс.

Материалы и методы. Осуществлена морфометрическая оценка суставного хряща (толщина, организация внеклеточного матрикса, пространственное расположение основных компонентов, плотность распределения, основные клеточные индексы хондроцитов) и изменений субхондральной кости (наличие костных разрастаний в виде появления костных балок в базальном слое суставного хряща и наличия единичных остеофитов) у 30 крыс с моделью первичного ОА, индуцированного путем последовательного введения в полость сустава 0,5 мл дексаметазона (2 мг) и 1 мл 10%-й суспензии стерильного талька в изотоническом растворе натрия хлорида. Изучены гистологические препараты коленных суставов, окрашенные гематоксилином Майера и эозином, альциановым синим (pH 1,0 и 2,5) по Ван-Гизону, Массону и Маллори. Метаболические особенности хрящевой и костной тканей изучены путем определения в сыворотке крови лабораторных животных концентраций гиалуронана, остеокальцина и коллагена I типа.

Результаты. У крыс с ОА, индуцированным введением дексаметазона и талька, выявлено уменьшение на 50% толщины суставного хряща в его нагружаемых участках, нарушение пространственного распределения хондроцитов, снижение ($p < 0,01$) ядерно-цитоплазматического отношения хондроцитов до 0,3 и повышение в сыворотке крови концентраций гиалуронана ($p < 0,001$) до 110,2 нг/мл, фрагментов коллагена I типа ($p < 0,001$) до 217,9 нг/мл и остеокальцина ($p < 0,001$) до 231,1 нг/мл.

Заключение. Основными патогенетическими особенностями экспериментального остеоартроза, индуцированного дексаметазоном и тальком, являются нарушение плотности распределения, морфологических характеристик и функциональной активности хондроцитов, что приводит к угнетению синтеза компонентов внеклеточного матрикса суставного хряща, а также сопровождается активизацией деструкции протеогликанов, содержащих несульфатированные гликозаминогликаны. Особенностью ремоделирования субхондральной кости при экспериментальном ОА, индуцированном дексаметазоном и тальком, является интенсификация синтетической активности остеобластов.

Ключевые слова: остеоартроз, крысы, дексаметазон, суставной хрящ, хондроциты, остеокальцин, субхондральная кость, гиалуроновая кислота, коллаген I типа

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INTRODUCTION

Osteoarthrosis (OA) is a common group of polyetiologic musculoskeletal diseases that progress over time and cause permanent changes in skeletal connective tissues [1]. Studying the pathogenetic mechanisms of the progress of inflammatory and destructive changes in the joints on animal models is aimed at enhancing available diagnostic and treatment strategies and designing new ones for further implementation of the obtained results into clinical practice [2, 3].

Commonly recognized methodological approaches to the experimental modeling of chronic joint disorders closely approximated to human OA in their morphology and involving all joint components (subchondral bone, articular cartilage, articular capsule and ligaments, as well as skeletal muscles that affect the joint) are based on the following major trends: breeding of animals of the same species with genetically determined OA, surgical destabilization of the knee joint components by resection of the anterior cruciate ligament or dissection of the menisci; affecting lubrication properties of the synovial fluid by administering abrasive solutions into the joint cavity, direct invasive effect of physical factors [4].

Initiation of degenerative and dystrophic changes in the articular cartilage through local application of various chemical agents, such as steroid medications (intraarticular administration of hydrocortisone acetate at a dose of 500 mg /kg of body weight, weekly intramuscular injections of dexamethasone at various doses depending on the animal species and duration of the experiment: 3 mg / kg, 7 mg / kg, or 10 mg / kg of body weight) is also widespread. The administration of other biologically active substances (intraarticular injections of vitamin A, monoiodoacetic acid in isotonic natrium chloride solution, 1% papain solution) also causes irreversible degenerative and destructive changes in the articular cartilage and a metabolic imbalance in the subchondral bone [5].

The mentioned methods of OA modeling result in quick (within 4–6 weeks after the manipulation) progression of damage to the joint structures similar to OA manifestations in humans. However, in animals, the most feasible methods of OA remodeling are comprehensive complex techniques based on a simultaneous targeted effect of a few harmful factors on joint tissues, reproducing various pathogenetic links of this chronic musculoskeletal pathology. In particular, this experimental trend is represented by local administration of 0.5 ml dexamethasone (2 mg) followed by intraarticular administration of 10% aqueous suspension of sterile talc into the articular cavity of the knee joint. This causes changes similar to stage I–II deforming arthrosis in humans [6].

Although experimental OA can be modeled by various methods [7], the morphology and metabolic features of articular tissues are similar and characterized by changes in the normal multilayer structure of the articular cartilage and impaired synthetic activity of chondroblasts with respect to aggrecan and lubricin that ensures viscosity of the synovial fluid. Moreover, in experimental OA, signs of hypercellularity in the basal layer of the articular cartilage, as well as structural and functional disorders in the articular cartilage – subchondral bone system are revealed [8].

There are studies confirming that along with uneven distribution of chondrocytes in various topographic areas of the articular cartilage, there are significant morphologic changes in chondrocytes characterized by karyopyknosis, karyolysis, and dystrophy leading to profound general changes in the metabolic activity of chondrocytes with respect to the main components of the extracellular matrix (ECM) and synovial fluid [9]. It was established that hyaluronan functions as a key protector and structural framework in providing appropriate microarchitecture of the articular cartilage under physiologic conditions along with proteoglycans (PG). This hyaluronic acid (HA) derivative is directly involved in stabilization and spatial arrangement of carbohydrate – protein complexes in the articular cartilage ECM. Besides, HA and its derivatives to-

gether with lubricin participate in cartilage homeostasis by affecting the diffusion and loading mechanism of articular tropism, as well as regulation of cell proliferation and migration, which proves their key role in maintaining the redox balance in articular structures [6].

However, opinions on the features of HA metabolism and its role in skeletal connective tissue remodeling in OA, especially in its early stages, differ. According to some studies, one of the key pathogenetic mechanisms in progression of damage to articular structures in OA is inhibition of HA synthesis against the background of increased hyaluronidase activity [10]. Some authors suggest that accumulation of HA in organs and tissues in OA results from an imbalance of its metabolism characterized by activation of its synthesis, as well as by functional and structural inconsistency of unsulfated glycosaminoglycans (GAG) formed following metabolic disturbances [11]. Therefore, we initiated a study aimed at investigating the structural and metabolic features of chondral and bone tissue remodeling in early manifestations of simulated knee OA.

The aim of the study was to investigate the pathogenesis of experimental OA induced by dexamethasone and talc by examining the structure and defining the morphometric and metabolic features of knee joint skeletal connective tissues in rats.

MATERIALS AND METHODS

The study was carried out in compliance with the principles of humanity set out in the directives of the European Community (86 /609 / EC), the Declaration of Helsinki, and "Rules for carrying out work using experimental animals" (Appendix to the order of the Ministry of Health of the USSR No. 755 dated 12.08.1977) and approved by the local Ethics Committee at Saratov State Medical University named after V.I. Razumovsky (Protocol No. 2 of 02.10.2018).

The study included 30 white outbred male rats aged 18 months and weighing 180–210 g. The animals were fed with standard wet food with free access to water and food. The animals were randomly divided into 2 groups: the control group included 10 intact rats, while the treatment group encompassed 20 animals. The animals of the treatment group had primary OA simulated in their knee joints by sequential administration of 0.5 ml dexamethasone (2 mg) into the cavity of their right (experimental) knee joint followed by 1 ml of 10% aqueous suspension of sterile talc a day later. 0.5 ml of isotonic sodium chloride solution was administered into their left (control) knee joint. Four weeks after the administration, the experimental OA had formed in the right knee joint of the animals corresponding to stage II–III deforming arthrosis in humans [4].

The animals were kept under observation for 4 weeks. The free movement amplitude was regularly evaluated, and the local status of the knee was defined. Local temperature was measured with infrared LAICA SA5900 (Italy) thermometer. Serum concentrations of hyaluronan, the key structural unsulfated glycosaminoglycan in the articular hyaline cartilage ECM, were determined by the end of the experiment by enzyme-linked immunosorbent assay (ELISA) using Quantikine® Hyaluronan Immunoassay (American Diagnostic Inc., USA). The findings were interpreted on the EPOCH™ spectrophotometer (BioTek Instruments, USA). Bone metabolism was assessed with reference to serum concentrations of type I collagen fragments found by ELISA using RatLaps™ enzyme-immunoassay (EIA) kits (Immunodiagnostic Systems Holdings Ltd, UK), as well as osteocalcin as a marker of osteoblast activity using Rat-MID™ Osteocalcin EIA kits (Immunodiagnostic Systems Holdings Ltd, UK).

After the rats were sacrificed, their knee joints were isolated as single osseomuscular specimens to study the features of their structure. The osseomuscular specimens were placed in 10% formalin, processed routinely, and embedded in paraffin. The histologic sections were cut and stained with Mayer′s hematoxylin (BioVitrum, Russia) and eosin (BioVitrum, Russia). The sections were mounted in the Bio Mount medium (Bio-Optica, Italy). To reveal histologic signs of OA, 8–10 7–10 μm thick frontal sections of the knee joints were used, cut at about 200 μm intervals in the areas of interest (lateral and medial femoral condyles, medial and lateral tibial plateaus). To assess the histologic changes in the knee joints, a semiquantitative scale was used [12].

The morphometric assessment of the histologic specimens (changes in particular areas of the articular cartilage and calculation of the nuclear – cyto-

plasmic (N / C) ratio for the main types of chondrocytes) was performed considering the cell area (*Sc*, μm²) and the nucleus area (Sn, μm²) in both groups of the animals. The N / C ratio was calculated using the following formula: N / C = Sn /(Sc – Sn), the measurements were taken using the Axio Imager Z2 microscope (Carl Zeiss, Germany).

The morphometric data were analyzed using digital images of the articular cartilage and subchondral bone in the areas of interest corresponding to the research objectives. The average number of cell elements was calculated in 6 fields of view in no less than 6 knee joint specimens for each topography area, and then the mean value was calculated. The sections were additionally stained with Alcian blue to reveal GAGs in the histologic specimens. Highly sulfated (pH 1.0) and total (pH 2.5) GAGs, as well as collagen fibers, underwent van Gieson's and Masson's staining, and collagen fibers were identified by van Gieson's and Mallory's staining.

Histologic specimens of the knee joints obtained from intact animals were used as controls. The animals were anesthetized with a combination of 0.05 ml / kg Zoletil-100 (Virbac Sante Animale, France) and 1 ml / kg Xylazine (Interchemie, the Netherlands) according to the instructions ("Veterinary Medicines in Russia" Guidelines, 2015). The animals were sacrificed by a 200 mg / kg overdose of Zoletil-100.

The findings were statistically processed using Statistica 10.0 software (StatSoft Inc, USA). All variables were tested using the Kolmogorov – Smirnov and Shapiro – Wilk tests. The retrieved values for each criterion suggested non-normal distribution of the data. As the findings did not correspond to the normal distribution, they were evaluated using the nonparametric Mann – Whitney U-test. The findings were presented as the median and the interquartile range *Me* (Q_1; Q_3). The differences between the animals with OA and the controls were considered statistically significant at $p < 0.05$.

RESULTS

The thickness of the articular cartilage in the weight-bearing areas of the femoral condyles in the controls (Table 1) was on average 220–300 μm, the articular cartilage contained mostly oval chondrocytes 11–18 μm in diameter with centrally located, normochromal nucleus, N / C 0.64 – type I chondrocytes. These cells were arranged both separately and in isogenous groups of 3–4 to 6 chondrocytes (Fig. 1).

Table 1

Morphometric features of the articular cartilage of the knee in the rats of the treatment and control groups, *Me* (Q_1; Q_3)

Group	Measured parameter		
	The thickness of the articular cartilage in the non-weight-bearing areas of the tibiae, mm	The thickness of articular cartilage in the weight-bearing areas of the femoral condyles, mm	The difference in the thickness of articular cartilages in weight-bearing and non-weight-bearing topography areas, %
Control group, $n = 10$	0.234 (0.221; 0.297)	0.226 (0.196; 0.281)	4.0 (0.9; 7.1)
Treatment group, $n = 20$	0.185* (0.209; 0.273) $p < 0.05$	0.105* (0.095; 0.125) $p < 0.01$	54.2 (22.4;67.1) * $p < 0.001$

* here and in Table 2, the differences between the parameters of the treatment and control groups at $p < 0.05$

The number of isogenous groups within one field of view was close to 10–11. Type II chondrocytes were located mostly in the basal layer of the articular cartilage, they were 8–9 μm in diameter, had centrally located pyknotic nuclei, and their N / C ratio did not exceed 0.30. There were 39–47 cells in each of the selected fields of view in the areas of interest, 70% of them were type I chondrocytes. In the superficial layer of the articular cartilage, cells were flattened and spindle-shaped with normochromic, bean-shaped nuclei.

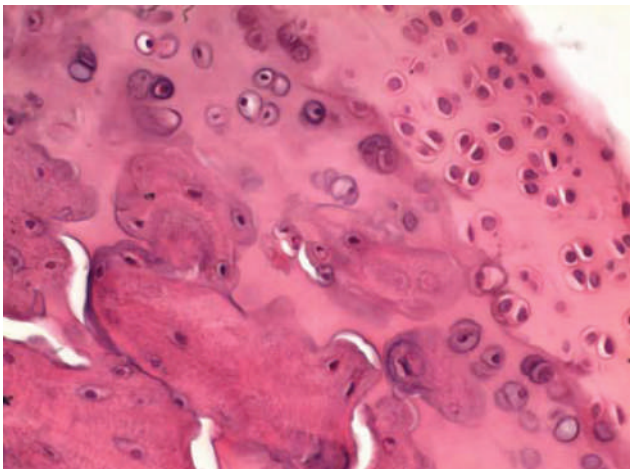

Fig. 1. The section of the articular cartilage in the knee of the intact control rat:hematoxylin – eosin staining, ×40

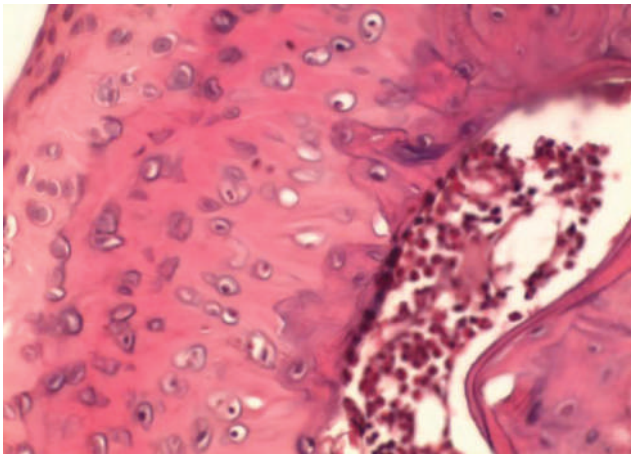

Fig. 2. The section of the articular cartilage in the knee of the rat from the treatment group: hematoxylin – eosin staining, ×40

4 weeks after the start of OA simulations, a decrease in the articular cartilage thickness ($p < 0.01$) in the weight-bearing areas was observed in the rats of the treatment group (Fig. 2). We also revealed changes in the proportion of chondrocytes, as well as in their morphology. The relative count of type I chondrocytes decreased and made about 40–50% of all articular cartilage cells. Their diameter did not exceed 10–13 μm, and their N / C ratio was close to 0.44. In some type I chondrocytes, the nuclei were heterochromatic and took an irregular bean shape, their arrangement was eccentric, and their cytoplasms had vague contours and triangular, spindle-like, or irregular bean-like shapes.

Type I chondrocytes formed a small number of isogenous groups (1–2 in the field of view), and each isogenous group comprised no more than 2–3 type I chondrocytes. About 22% of type II chondrocytes were characterized by pronounced karyopyknosis and eccentric arrangement of their nuclei, and 70% of all type II chondrocytes were characterized by microcytosis (4–6 μm in diameter). Enucleated forms of type II chondrocytes (karyolysis) made up about 40% of all chondrocytes. Individual acellular lacunae were found in the articular cartilage. Around 27–30 isolated or clustered chondrocytes were observed in each field of view. We also observed some individual trabeculae in the basal layer of the articular cartilage, which had a regular round shape in their cross-section.

Additional staining methods allowed to detect a decrease in the levels of all analyzed GAGs in the organic ECM of the articular cartilage in the animals of the treatment group. This indicated profound degenerative and dystrophic processes and was proven by biochemical findings. Thus, the study of the key cartilage and bone metabolites in the serum (Table 2) revealed a significant increase in the concentrations of type I collagen fragments ($p < 0.001$) and hyaluronan ($p < 0.001$) in the animals of the treatment group compared with the controls. Osteocalcin supply to the blood of OA rats ($p < 0.001$) decreased suggesting signs of inhibited synthetic activity in osteoblasts.

Table 2

Serum concentrations of hyaluronan, type I collagen fragments, and osteocalcin in the rats with simulated primary OA, *Me* (Q_1; Q_3)

Group	Serum concentrations		
	Hyaluronan, ng / ml	Type I collagen fragments, ng / ml	Osteocalcin, ng / ml
Controls, $n = 10$	66.5 (57.6; 69.9)	144.2 (99.3; 170.1)	231.5 (222.9; 258.4)
Treatment group, $n = 20$	110.2* (81.4; 142.9) $p < 0.001$	217.9* (200.5; 248.2) $p < 0.001$	176.9* (133.1; 194.4) $p < 0.001$

DISCUSSION

Under physiological conditions, the metabolism of skeletal connective tissues is characterized by the balance between catabolic and anabolic processes, which provides structural and functional integrity of the knee joint [13]. Cells, such as synoviocytes, mononucleotides that infiltrate the synovial membrane, osteocytes in the subchondral bone, and chondrocytes, play an essential role in healthy skeletal connective tissue remodeling [3].

The articular cartilage is a complex structural and functional system of cells and extracellular matrix (fibers and amorphous core). However, the major biomechanical features of the articular cartilage are determined by high aggrecan hydration, while degenerative and dystrophic changes in OA result from several unfavorable exogenous and endogenous effects leading to an evident decrease in cartilage congruence [1, 11].

The conducted morphology tests revealed the progression of irreversible degenerative and dystrophic changes in chondrocytes in simulated OA,

manifested through impaired cell ratios, proliferation, and death. The changes observed in simulated OA suggested that the imbalance of metabolic processes in the articular cartilage ECM resulted from its structural and functional modification (reduction of chondrocyte density, their rearrangement, changes in the N / C ratio, signs of cell death). This was confirmed by the increase in serum hyaluronan concentrations in the rats of the treatment group. These changes probably result from the prevalence of destructive changes aimed at disorganizing proteoglycan complexes of intercellular substance in the articular cartilage. The imbalance between catabolic and anabolic responses in the articular cartilage is confirmed by a significant decrease in its thickness in simulated OA.

Disorders in the subchondral bone are definitely some of the key factors in the OA pathogenesis. Under normal conditions, they ensure essential needs of the articular cartilage, including its densification, hardening, and osteophyte formation with changes in the general biomechanics of joints and active penetration of inflammatory mediators and growth factors in response to enhanced angiogenesis [14, 15]. Our findings revealed the activation of osteogenesis following the increase in osteoblast secretion and alteration of their metabolism, which was confirmed by the increase in serum osteocalcin levels in the rats of the treatment group in early signs of OA.

CONCLUSION

The main pathogenetic features of experimental OA induced by dexamethasone and talc include impaired distribution density, morphological characteristics, and functional activity of chondrocytes, which results in inhibited synthesis of extracellular matrix components in the articular cartilage and activated destruction in supramolecular complexes containing unsulfated GAGs. Disturbances of subchondral bone remodeling in experimental OA are characterized by intensification of synthetic activity in osteoblasts.

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Relationship of parameters of the impulsivity – reflexivity cognitive style with propensity to Internet addiction in high school students

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ABSTRACT

Aim. To study the relationship between parameters of the impulsivity – reflexivity cognitive style and the level of Internet addiction among high school students.

Materials and methods. 154 students from general education institutions aged 16–17 years were diagnosed using the J. Kagan's reflection – impulsivity test and the Chen Internet Addiction Scale. The one-way analysis of variance was used, followed by post-hoc pairwise comparisons using the Tukey's test.

Results. An inverse relationship between the level of Internet addiction and decision latency in a multiple-choice situation and a direct relationship between the level of Internet addiction and the number of errors in the J. Kagan's reflection – impulsivity test were revealed.

Conclusion. High school students with propensity to Internet addiction tend to have the impulsive cognitive style, while students without such a tendency – the reflective style. In cognitive activity, the impulsive cognitive style is generally less productive than the reflexive one. The impulsive cognitive style is similar to the so-called clip thinking, which allows to consider the relationship between Internet addiction and this phenomenon through the prism of cognitive and style features.

Keywords: Internet addiction, high school students, impulsivity – reflexivity cognitive style

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

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Связь показателей когнитивного стиля «импульсивность/рефлексивность» со степенью склонности к интернет-зависимому поведению у старшеклассников

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РЕЗЮМЕ

Цель – изучение связи характеристик когнитивного стиля «импульсивность/рефлексивность» с уровнем интернет-зависимости у старшеклассников.

Материалы и методы. Проведена диагностика 154 обучающихся общеобразовательных учреждений 16–17 лет посредством теста Дж. Кагана для определения показателей когнитивного стиля «импульсивность/рефлексивность» и теста «Шкала склонности к интернет-зависимому поведению» С. Чена. Использовался однофакторный дисперсионный анализ с последующими попарными апостериорными сравнениями критерием Тьюки.

Результаты. Установлено наличие обратной связи между уровнем интернет-зависимости и латентным временем принятия решения в ситуации множественного выбора и прямой связи между уровнем интернет-зависимости и количеством ошибок в тесте Дж. Кагана.

Заключение. Старшеклассники со склонностью к интернет-зависимости тяготеют к импульсивному типу по исследованному когнитивному стилю, без таковой склонности – к рефлексивному типу. В познавательной деятельности использование импульсивного когнитивного стиля в целом менее продуктивно, чем рефлексивного. Импульсивный когнитивный стиль обнаруживает черты сходства с так называемым клиповым мышлением, что позволяет рассматривать связь интернет-зависимости с этим явлением через призму когнитивно-стилевых особенностей.

Ключевые слова: интернет-зависимость, старшеклассники, когнитивный стиль «импульсивность/рефлексивность»

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

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Соответствие принципам этики. Все испытуемые подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом ФИЦКИА им. акад. Н.П. Лаверова УрО РАН (протокол № 3 от 12.02.2020).

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INTRODUCTION

The phenomenon of Internet addiction (IA) is widely studied all over the world [1–3]. Various aspects of the impact of Internet addiction on mental [4] and physical health [5] are being investigated, especially in adolescents. A steady increase in the time spent on the Internet is inevitable due to sociocultural processes, as well as an increasing tilt of education and many types of professional activity to the online environment [6]. Uncontrolled use of the Internet, especially social networks, and viewing content not related to professional or educational activities is a powerful addictive factor. Internet addiction already takes its place among other addictions and is difficult to correct [7, 8]. A recent Russian study of 3,012 adolescents aged 12–18 years showed that maladaptive use of the Internet is typical of 43.6% of adolescents, and the incidence of Internet addiction increases with age [9].

Along with the increase in Internet addiction among the population, the phenomenon of the so-called clip thinking is becoming more and more pronounced, especially among the younger generation, who have been active Internet users since childhood [10]. At the same time, there are practically no generally accepted methods for diagnosing clip thinking; variants of its detection are also very vague and diverse [11, 12]. However, all researchers of clip thinking, one way or another, admit that its main features include perception of information through bright, short, often unrelated images, fast switching from one topic to another, and a need for constant information updates. All these factors combined result in problems with concentration, inability to analyse information and retain it in memory, misunderstanding of the meaning of what was read, increased suggestibility, etc.

It seems promising to investigate a relationship of Internet addiction, first of all, with the parameters of the impulsivity – reflexivity cognitive style [13], because it is individuals with the impulsive cognitive style that, in our opinion, show some similarity with people characterized by clip thinking. This task is especially relevant for high school students [14], who, on the one hand, have to increasingly use the Internet for education, and on the other hand, are constantly faced with all sorts of temptations to spend time on the Internet on unproductive activities with addictive potential.

The aim of this research was to study the relationship between parameters of the impulsivity – reflexivity cognitive style and the level of Internet addiction among high school students.

MATERIALS AND METHODS

The subjects of the study were students of general education schools aged 16–17 years from the cities of Simferopol (99 people, 32 males and 67 females) and Nadym (55 people, 17 males and 38 females).

As a tool for diagnosing Internet addiction, we used the Chen Internet Addiction Scale adapted by K.A. Feklisov and V.L. Malygin. The parameters of the impulsivity – reflexivity cognitive style were diagnosed using J. Kagan's "Comparison of Similar Drawings" test. The subject of the study was presented with 2 training sheets, and then with 12 basic sheets: on top of the sheet, there was an image of a familiar object (reference figure), at the bottom, there were 8 practically identical images of the same object arranged in two rows, among which only one fully corresponded to the reference figure. The subject had to find and indicate an image that was completely identical to the reference figure. The following parameters were recorded: 1) the average decision latency of the first response in 12 series (in seconds); 2) the total number of errors made in the test.

The Kolmogorov – Smirnov test was used to check the data series for normal distribution. Both for the total sample and for the 6 compared subsamples, no statistically significant differences in deviations of the data series from the normal distribution were found (Table). For the smallest sample, which comprised 20 variants, the more sensitive Shapiro – Wilk test was used to check for normality of distribution.

Since in none of the cases the statistical distribution of the data differed from the normal distribution, a one-way analysis of variance (ANOVA) was used, followed by post-hoc pairwise comparisons using the Tukey's test. Statistical processing was performed using the Statsoft Statistica 13 software (StatSoft Inc., USA).

Table

Results of testing data series for normality of distribution*

Samples	"Response time" variable	"Number of errors" variable
Total sample, *n* = 154	K–S d = 0.090	K–S d = 0.082
IA is absent, *n* = 52	K–S d = 0.086	K–S d = 0.110
Propensity to IA, *n* = 82	K–S d = 0.130	K–S d = 0.094
Pronounced IA, *n* = 20	S–W W = 0.94	S–W W = 0.98

*$p > 0.05$.

RESULTS

First, we performed ANOVA for the "response time" variable. Highly significant differences were revealed in the distribution of the trait in the subsamples according to the Fisher's criterion: F (2, 151) = 10.21, $p = 0.00007$. The analysis results are graphically presented in Fig. 1.

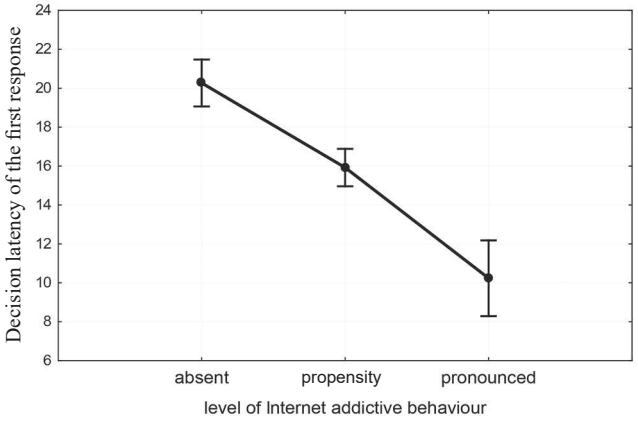

Fig. 1. ANOVA findings for the relationship between the decision latency of the first response in the J. Kagan's test and propensity to Internet addiction

A close to inverse relationship between the decision latency of the first response (response time) and propensity to Internet addiction was revealed. Thus, for individuals without IA, the response time was 20.27 ± 1.21 sec, for those with propensity to IA – 15.92 ± 0.96 sec, and for those with pronounced IA – 10.24 ± 1.96 sec.

Further analysis of statistical significance using the Tukey's honest significance test showed the presence of statistically significant differences between all the subgroups:

between individuals with no IA and those with propensity to IA, $p = 0.013$;

between individuals with no IA and those with pronounced IA, $p = 0.000055$;

between individuals with propensity to IA and those with pronounced IA, $p = 0.024$.

Thus, all IA levels are significantly different from each other in terms of decision latency and form a close to linear trend toward a decrease in the decision latency as propensity to Internet addiction increases.

Next, we analysed the distribution of the "number of errors" variable depending on propensity to IA. Significant differences in the distribution of the variable in the subsamples were revealed according to the Fisher's criterion: F(2, 151) = 4.4452, $p = 0.013$. The analysis findings are graphically presented in Fig. 2.

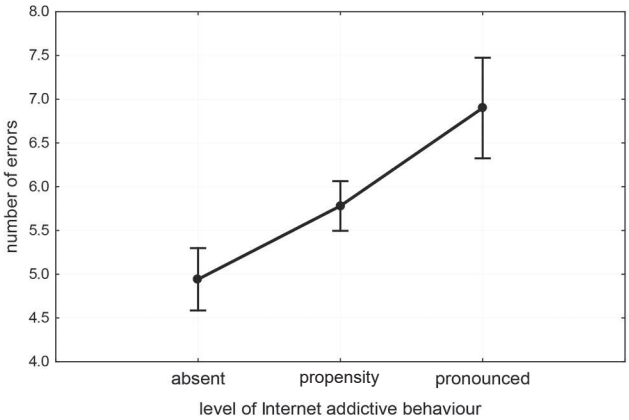

Fig. 2. ANOVA findings for the relationship between the number of errors in the J. Kagan's test and propensity to Internet addiction

For this variable, a close to inverse relationship between the number of errors in the test and the level of IA was revealed.

Thus, in individuals without IA, the number of errors was 4.94 ± 0.36, in those with propensity to IA – 5.78 ± 0.28, and in those with pronounced IA – 6.90 ± 0.57.

Further analysis of statistical significance using the Tukey's post hoc test showed the following levels of significance of the differences:

1) between individuals with no IA and those with propensity to IA: $p = 0.15$;

2) between individuals with no IA and those with pronounced IA: $p = 0.010$;

3) between individuals with propensity to IA and those with pronounced IA: $p = 0.18$.

The significant differences in the "number of errors" parameter were observed only between individuals without IA and those with it, while in individuals with propensity to IA no significant differences in the

"number of errors" parameter were revealed compared with the two mentioned subgroups. Nevertheless, there is a clear upward trend in the "number of errors" parameter as Internet addiction increases.

Summarizing the preliminary results, it can be stated that individuals with IA are less accurate, but faster in making decisions, which makes them similar to individuals with the impulsive cognitive style. On the contrary, individuals with IA are more similar to the reflexive cognitive style due to longer deliberation of a task with greater accuracy of answers.

DISCUSSION

In the present study, new data were obtained on the relationship between IA and the parameters of the impulsivity – reflexivity cognitive style: the higher the level of IA is, the faster the subject makes a decision in a situation of multiple cognitive choice, making more mistakes. On the contrary, the lower the level of IA is, the longer the subject thinks over the solutions, but the more accurate they are.

The J. Kagan's test does not contain criteria for assigning individuals to a particular cognitive style; it is only recommended to rely on medians in a particular sample when making an appropriate psychodiagnostic conclusion. However, from the point of view of psychodiagnostics, this approach is not sufficiently rigorous, since the data can vary greatly from sample to sample for various reasons, and the population for standardization must include at least an order of magnitude more test subjects. At the same time, the trends we observe show that individuals without propensity to IA are apparently closer to the reflexive cognitive style, that is, they make relatively few mistakes, but use a relatively longer time to think over tasks; conversely, individuals with pronounced IA make more mistakes with less thinking time.

The nature of cognitive styles is not fully elucidated. On the one hand, they are associated with temperament and features of interhemispheric asymmetry. On the other hand, they can change to a certain extent during the lifetime under the influence of cultural, social, and psychological factors [14]. There is an opinion that cognitive styles are stable with time, characterizing the features of individual's cognitive activity [15, 16]. However, there is substantiated evidence that cognitive styles are formed *in vivo* in the process of active life [17]. In [18], the analysis of numerous works is presented on the fact that any cognitive tasks are more effectively solved by reflexive individuals. Polar differences have been established between reflexive and impulsive individuals in terms of emotionality and activity: reflexive individuals are less emotional and more active; impulsive ones are more emotional and less active. Therefore, impulsive individuals show the first spontaneous reaction in solving the problem emotionally, and the reflexive ones, having more energy, work out additional options for solving problems instead of experiencing strong emotions [19]. At the same time, since persons characterized by the impulsive cognitive style not only make decisions in an insufficiently meaningful way, but are generally characterized by low self-control [20], they are more prone to IA than persons with greater reflexivity.

IA is a powerful factor influencing cognitive processes, including the ones at the brain level [21], and the results of our study suggest that the level of IA may somehow influence the cognitive styles. This highlights the problem of IA even more and requires engagement of different specialists in its solution.

CONCLUSION

The degree of propensity to IA is associated with characteristics of the impulsivity – reflexivity cognitive style. According to the parameters of the J. Kagan's test, individuals with pronounced IA are characterized by shorter decision latency in a cognitive task with a greater number of errors, while individuals without IA tend to take longer time to make a decision with fewer errors. At the same time, individuals with propensity to IA occupy an intermediate position according to the parameters of the J. Kagan's test. The results obtained, to some extent, clarify the phenomenon of clip thinking (fast, shallow, emotional, non-analytical), linking it both with the degree of IA and with a tilt to the impulsive cognitive style.

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Markers of kidney injury, lipid metabolism, and carbonyl stress in patients with type 1 diabetes and different levels of albuminuria

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ABSTRACT

The **aim** of this work was to study the levels of podocalyxin and β-2-microglobulin and parameters of lipid metabolism and carbonyl stress in type 1 diabetes mellitus (T1DM) patients with different levels of albuminuria.

Materials and methods. 56 men of reproductive age with T1DM were divided into two groups: 24 patients with stage A1 albuminuria (group A1) and 32 patients with stage A2 albuminuria (group A2). The control group consisted of 28 healthy men. The levels of renal function markers, lipid metabolism parameters, and methylglyoxal were assessed using enzyme immunoassay and spectrophotometric and fluorometric methods.

Results. Higher values for total cholesterol, triacylglycerol, and very-low-density lipoprotein medians in both groups A1 and A2 were found. In these groups, increased podocalyxin and methylglyoxal medians were revealed. Correlation analysis in the group A1 showed the presence of a relationship between the glomerular filtration rate (GFR) and creatinine. In the group A2, correlations between the generally accepted parameters of kidney injury (the albumin / creatinine ratio and GFR) and the duration of the disease and between GFR and the creatinine and methylglyoxal levels in the blood were identified. The podocalyxin level in this group correlated with the β2-microglobulin and methylglyoxal levels and lipid metabolism parameters. The level of β2-microglobulin correlated with the lipid metabolism parameters.

Conclusion. Regardless of the level of albuminuria, men with T1DM had significantly increased levels of podocalyxin, lipid metabolism parameters, and methylglyoxal, as well as strong relationships between these parameters. The data of this study can be used for development of potential strategies for prevention and early treatment of diabetic nephropathy.

Keywords: type 1 diabetes mellitus, men, albuminuria, podocalyxin, β-2-microglobulin, carbonyl stress, lipids

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Маркеры почечного повреждения, липидного обмена и карбонильного стресса у пациентов с сахарным диабетом I типа и разным уровнем альбуминурии

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РЕЗЮМЕ

Цель – изучение уровня подокаликсина, β-2-микроглобулина, показателей липидного обмена и карбонильного стресса у пациентов с сахарным диабетом (СД) I типа и разным уровнем альбуминурии.

Материалы и методы. Проведено обследование 56 мужчин репродуктивного возраста с СД I типа, разделенных на две группы: 24 пациента с альбуминурией стадии А1 (группа А1) и 32 – с альбуминурией стадии А2 (группа А2). Контрольную группу составили 28 здоровых мужчин. Оценивался уровень почечных маркеров, компонентов липидного обмена и метилглиоксаля (МГ) с использованием иммуноферментных, спектрофотометрических и флюорометрических методов.

Результаты. Установлены более высокие значения медиан общего холестерина, триацилглицеридов и липопротеидов очень низкой плотности в обеих группах с СД I типа. В данных группах отмечались также повышенные значения медианы подокаликсина и основного показателя карбонильного стресса – МГ. Проведенный корреляционный анализ в группе А1 показал наличие зависимости уровня скорости клубочковой фильтрации (СКФ) и креатинина. В группе А2 отмечались связи общепринятых показателей почечного повреждения (соотношения альбумин/креатинин и СКФ) с длительностью заболевания, показателя СКФ с уровнем креатинина и МГ. Уровень подокаликсина в данной группе коррелировал с уровнем β2-микроглобулина, МГ, показателей липидного обмена; β2-микроглобулин имел взаимосвязи с параметрами липидного обмена.

Заключение. У мужчин с СД I типа вне зависимости от уровня альбуминурии отмечаются значительно более высокий уровень подокаликсина, увеличенные показатели липидного обмена и МГ, а также наличие тесных взаимосвязей между этими параметрами, что может быть использовано для разработки потенциальных стратегий профилактики и ранней терапии диабетической нефропатии.

Ключевые слова: сахарный диабет I типа, мужчины, альбуминурия, подокаликсин, β-2-микроглобулин, карбонильный стресс, липиды

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

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INTRODUCTION

Diabetes mellitus (DM) is considered a pressing problem due to the development of numerous complications. Diabetic nephropathy (DN) occupies a prominent place among the DM complications due to early disability and mortality [1, 2]. The incidence of DN among patients with type 1 diabetes mellitus (T1DM) is 20.1% [3]. DN is a complex lesion of the arteries, arterioles, glomeruli, and tubules of the kidneys, often leading to the development of diffuse or nodular glomerulosclerosis and, subsequently, to chronic renal failure [4].

Multiple factors contribute to the DN development, including metabolic (hyperglycemia, hyperlipidemia) and hemodynamic factors [2, 4]. Poor glycemic control and hereditary predisposition contribute to the progression of DN[1]. Hyperglycemia can adversely affect renal structures through a number of factors, such as activation of the sorbitol pathway of glucose metabolism, increased synthesis of diacylglycerol, accumulation of non-enzymatic glycation products of proteins and lipids in tissues, etc. [5, 6]. It is commonly accepted to differentiate several stages in DN development; however, it was established that changes in kidney tissues in patients with diabetes occur already when excretion of albumin in the urine is normal [7]. For this reason, it becomes especially important to clarify the mechanisms that contribute to early changes in renal structures in DM patients. Currently, the main groups of new potential renal markers have been identified: tubular markers, markers of podocyte damage, growth factors, immune and inflammatory factors, and products of extracellular matrix metabolism, that allow to predict the development of DN with high specificity and sensitivity [8].

Carbonyl stress is a condition that is accompanied by an increase in the content of carbonyl compounds, such as aldehydes, ketones, carboxylic acids, carbohydrates, etc. Most of the compounds of such nature are aldehydes: malondialdehyde, 4-hydroxynonenal, glyoxal, methylglyoxal, acrylic aldehyde, etc. [9]. Most aldehyde synthesis reactions involve free radicals or free radical oxidation products, which determines a close causal relationship between carbonyl and oxidative stress [10]. Currently, it has been shown that carbonyl compounds can accumulate in the body for a long period of time in DM, which together with additional pathogenetic mechanisms leads to serious dysregulations in the kidneys [11, 12].

Despite the available research data, there is still insufficient knowledge about the relationship between various factors of kidney injury and carbonyl stress parameters in the T1DM development. Therefore, the aim of this study was to investigate the levels of podocalyxin, β-2-microglobulin, and lipid metabolism and carbonyl stress parameters in T1DM patients with different levels of albuminuria.

MATERIALS AND METHODS

The data of 56 T1DM patients of young reproductive age (average age 30.25 ± 8.51 years) with a poor glycemic profile were used. According to the latest classification, this group was divided into 2 subgroups: patients with stage A1 albuminuria (group A1) (n = 24, average age 29.38 ± 9.78 years) and patients with stage A2 albuminuria (group A2) (n = 32, average age 30.88 ± 7.54 years) [13]. According to the disease duration, glycated hemoglobin level (HbA1c), and glycemic profile, the mean values in these groups did not differ from each other ($p > 0.05$).

The examination of patients included a comprehensive assessment of clinical and laboratory data. The glycemic profile (fasting blood glucose, postprandial glucose 2 hours after a meal) was assessed. The concentration of glycated hemoglobin (HbA1c) was determined by ion-exchange high-performance liquid chromatography, using a D-10 analyzer (Bio-Rad, USA). The following diagnostic methods were used to assess early kidney injury: glomerular filtration rate (GFR), albumin content, urinary albumin / creatinine ratio. The albumin content and the albumin / creatinine ratio in the urine were determined on the SYNCHRON CX9 PRO biochemical analyzer (Beckman Coulter, USA) using the immunoturbidimetric assay. GFR was calculated according to CKD – EPI equation (ml / min / 1.73 m^2).

The research materials were serum and urine. The level of podocalyxin in the urine was determined by enzyme immunoassay using the Podocalyxin ELISA Kit (USA). The level of β-2-microglobulin in the urine was determined using the Beta-2-microglobulin kit (BioChemMack, Russian Federation). The content of total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, and triacylglycerols (TG) in the blood serum was determined using the

Bio Systems commercial kits (Spain). Measurements were carried out on the SYNCHRON CX9 PRO biochemical analyzer (Beckman Coulter, USA). The level of very-low-density lipoprotein (VLDL) cholesterol was calculated using the following formula: VLDL = TG / 2.2; and low-density lipoprotein (LDL) cholesterol = TC – (HDL + VLDL). The content of methylglyoxal, a carbonyl stress parameter in the blood serum, was determined using the Human Methylglyoxal ELISA Kit (USA). Enzyme immunoassay was performed on the MultiSkan ELX808 microplate reader (Biotek, USA).

This study was carried out using the equipment of the Center for the Development of Progressive Personalized Health Technologies at Scientific Center for Family Health and Human Reproduction Problems (Irkutsk).

Statistica 8.0 package (StatSoft Inc., USA) was used for statistical processing of the obtained results. At the first stage, the normality of distribution was determined by the visual – graphic method and the Kolmogorov – Smirnov test with the correction using the Lilliefors and Shapiro – Wilk tests). The equality of generalized variance was checked using Fisher's exact test (F-test). Further, due to the difference between the sample and the normal distribution, the nonparametric Mann – Whitney test was used. The results were presented as the median and the interquartile range of $Me[Q_1–Q_3]$. The Spearman's rank correlation coefficient was used for correlation analysis. The differences were considered statistically significant at $p = 0.05$.

RESULTS

The analysis of serum lipid content in T1DM patients in the groups with different levels of albuminuria is presented in Table 1.

Table 1

Serum lipid content in T1DM patients with different levels of albuminuria, *Me* [Q_1–Q_3]

Parameter, mmol / l	Control group	Group A1	Group A2
TC	4.21 [3.74–4.58]	4.6 [4.15–5.18]*	4.65 [4.15–5.5]*
TG	0.66 [0.47–0.93]	1 [0.8–1.55]*	1.2 [0.8–1.8]*
HDL cholesterol	1.28 [1.00–1.4]	1.39 [1.1–1.5]	1.3 [1–1.7]
LDL cholesterol	2.47 [2.22–2.99]	2.44 [1.96–2.86]	2.37 [1.98–2.9]
VLDL cholesterol	0.3 [0.21–0.42]	0.46 [0.36–0.71]*	0.55 [0.36–0.82]*

* Here and in Table 2, statistically significant differences with the control group ($p < 0.05$).

According to the results, group A1 had higher median values of TC ($p = 0.005$), TG ($p = 0.007$), and VLDL cholesterol ($p = 0.007$) compared with the controls (Table 1). Group A2 also differed from the control values in higher TC ($p = 0.001$), TG ($p = 0.022$), and VLDL cholesterol ($p = 0.022$). No statistically significant differences ($p > 0.05$) were identified in the other parameters in the study groups (Table 1).

Urinary excretion levels of kidney injury markers were measured in groups A1 and A2 (Figure). Higher levels of podocalyxin were found in group A1 ($p = 0.003$) and group A2 ($p = 0.004$) compared with the control group. No significant differences were found concerning β2-microglobulin ($p > 0.05$).

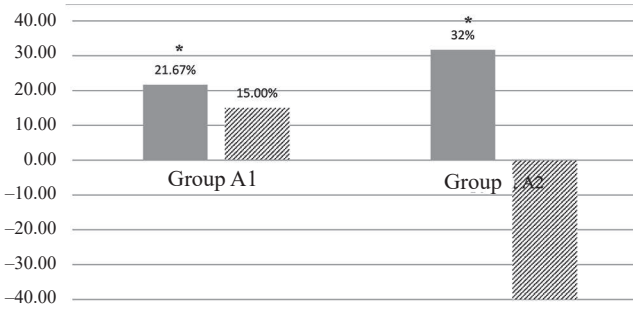

Figure. Urinary excretion levels of podocalyxin and β-microglobulin in patients with T1DM and different levels of albuminuria, %. * Statistically significant differences with the control group ($p < 0.05$). The control values are taken as 0%

Table 2 shows the results of assessing the concentration of serum methylglyoxal, a key carbonyl stress parameter, in patients with T1DM. It was found that higher median methylglyoxal values ($p = 0.031$) in group A1 were noted compared with the control. In group A2, similar differences were found ($p < 0.001$) compared with the control.

Table 2

Serum methylglyoxal concentration in patients with T1DM and different levels of albuminuria, *Me*[Q_1–Q_3]

Control group	Group A1	Group A2
2.14 [1.02–3.67]	3.24 [2.6–3.51]*	3.46 [2.9–4.21]*

The correlation analysis conducted in group A1 showed a single relationship between GFR and creatinine ($r = –0.79$; $p = 0.0001$). Group A2 was characterized by correlations between the duration of the disease and albumin / creatinine ratio ($r = 0.47$;

$p = 0.018$) and between GFR and blood creatinine level ($r = –0.44$; $p = 0.027$) and methylglyoxal level ($r = 0.64$; $p = 0.043$). Podocalyxin levels in this group correlated with the levels of another renal marker, β2-microglobulin ($r = 0.47$; $p = 0.018$), and with the methylglyoxal level ($r = 0.52$; $p = 0.008$). In addition, podocalyxin showed correlations with lipid metabolism parameters: TC ($r = 0.42$; $p = 0.036$), TG ($r = 0.41$; $p = 0.04$), VLDL cholesterol ($r = 0.41$; $p = 0.04$). β2-microglobulin had correlations with TC ($r = 0.52$; $p = 0.007$), TG ($r = 0.42$; $p = 0.035$), and VLDL cholesterol ($r = 0.42$; $p = 0.035$). In group A2, there were also multiple correlations of lipid metabolism parameters among themselves and with other parameters: TC – TG ($r = 0.62$; $p = 0.001$), TC – VLDL cholesterol ($r = 0.62$; $p= 0.001$), TG – VLDL cholesterol ($r = –0.6$; $p = 0.002$), LDL cholesterol – VLDL cholesterol ($r = –0.6$; $p = 0.002$), HDL cholesterol – creatinine ($r = –0.43$; $p = 0.032$).

DISCUSSION

Assessment of the serum lipid content in the groups showed a slight increase in TC, TG, and VLDL cholesterol in groups A1 and A2. Currently, hyperlipidemia is considered as a separate serious factor in DN progression due to the direct relationship of complex lipid disorders with the formation of glomerulosclerosis [4, 14]. In our study, there were no significant changes in the lipid content in both groups, although a definite trend was observed. Hyperlipidemia has recently been considered as a separate nephrotoxic factor, with a clear parallel drawn between the processes of glomerulosclerosis and vascular atherosclerosis [15]. It was found that oxidized LDL, growth factors, and cytokines increase synthesis of mesangial matrix components, accelerating glomerular sclerosis, which contributes to the progression of DN [4].

The analysis of the urinary excretion levels of podocalyxin and β-microglobulin in the study groups showed significant changes only for podocalyxin. We noted that its urinary excretion was elevated in both groups relative to the control, with higher excretion in group A2. Podocalyxin is a specific protein expressed on the surface of podocytes [16]. Experimental studies on models of DN formation showed that podocyte damage plays a crucial role in filtration barrier permeability disorders and glomerulosclerosis development, with significant podocyte desquamation into the urinary space [17].

Currently, the link between the number of podocytes in the urine and kidney diseases, such as nephropathy, systemic nephritis, focal segmental glomerulosclerosis, etc., has been convincingly proven [18]. This parameter reflects damage to the juxtaglomerular apparatus of the kidneys, with increased podocyte excretion in the urine [19]. Several studies showed that podocyturia develops in 74% of patients with normal albuminuria and in 54% of patients with microalbuminuria, with the same frequency in types 1 and 2 diabetes [20]. These data indicate that podocytes in DM are damaged much earlier than filtration barrier permeability disorders occur, i.e. in stage A2 albuminuria [21]. Thus, our data confirm previous studies on the increase in this parameter in patients with DN [19, 20].

Another parameter, β-microglobulin, showed no significant changes in both groups. This parameter characterizes damage to the renal tubules, and, thus, it can be stated that no pronounced changes of this kind were detected in the patients.

Methylglyoxal, a key carbonyl stress parameter, was elevated in T1DM patients of both groups. Methylglyoxal is a carbonyl compound, a precursor of glycotoxins formed by the non-enzymatic browning reaction [10, 12, 22]. Thus, under the conditions of chronic hyperglycemia, there is a significant increase in the intracellular glucose content, and pathological pathways of its metabolic transformation are activated along with insufficient utilization [6]. These processes, together with oxidative stress reactions, lead to the formation of stable Amadori products from reversible unstable Schiff bases. During the reactions, Amadori products are converted into fluorescent proteins, glycotoxins, and so-called advanced glycation end products (AGEs).

The latter were found to accumulate, slowly degrade, and persist for a long time in the vascular bed, even with further stabilization of glucose levels – a mechanism of metabolic memory [12, 23]. AGEs are involved in cross-linking of long-lived proteins, which contributes to arterial wall stiffening [23]. Their role was shown in the mitochondrial protein modification, impaired mitochondrial function, and overproduction of free radicals [10]. In this regard, methylglyoxal is considered as an important biomarker of diabetic complications due to

its close connection with glycation processes, β-cell dysfunction, and insulin resistance [24]. The amount of AGEs was found to be directly proportional to the level of blood glucose, and even a moderate increase in blood glucose leads to an increase in AGEs.

Methylglyoxal is also considered to be the most reactive among AGEs due to its direct involvement in disrupting insulin secretion and function, as well as in signal transduction processes. There is evidence that the intrinsic AGE receptor, RAGE, present on the cell surface, may serve as an important therapeutic target in DM patients with chronic kidney disease, and its blockade leads to a delay in the progression of vascular complications [22]. Thus, less functional damage to renal structures was observed in mice with AGE receptor knockout [23]. It was also found that increased AGE levels are closely associated with various structural and functional changes characteristic of DN, in particular with GFR. [12, 22]. Mitochondrial AGE formation is also thought to be an irreversible phenomenon underlying the metabolic memory mechanism through formation of reactive oxygen species, which in turn may contribute to the development of damaging effects in mitochondrial DNA and inhibition of the respiratory chain [24, 25].

Therefore, increased methylglyoxal concentrations in the blood of T1DM patients with microalbuminuria may indicate DM development, whereas under the conditions of stage A2 albuminuria, they may indicate a potential role of carbonyl stress in DN development. The correlation analysis in the group of patients with microalbuminuria revealed a regular relationship between GFR and creatinine. Under the conditions of stage A2 albuminuria, there were correlations between the generally accepted parameters of kidney injury (albumin / creatinine ratio and GFR) and the duration of the disease. Podocalyxin showed a close relationship with methylglyoxal, which may indicate a significant contribution of glycotoxins to the mechanisms of kidney injury during the development of albuminuria.

This fact was also confirmed by the revealed relationship of methylglyoxal with GFR. Although no changes in the mean values of β-microglobulin were detected in group A2, this parameter correlated with podocalyxin, which demonstrates the similarity of their damaging effects in different parts of the juxtaglomerular apparatus of the kidneys. The similarity of podocalyxin and β-microglobulin correlations with the lipid content (TC, TG, and VLDL cholesterol), which may be due to the significant contribution of these components to the DN progression, is of great interest.

CONCLUSION

It can be stated that men with T1DM have an increase in podocalyxin, which indicates damage to the juxtaglomerular apparatus of the kidneys. The development of carbonyl stress is also recorded. These disorders also apply to stage A1 albuminuria, when pronounced changes in the functioning of renal structures have not yet occurred. This confirms the suggestion that even in early stages of the disease there are conditions for the activation of adverse factors and progression of diabetic complications. This can be used to develop potential strategies for prevention and therapy of DN.

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Authors contribution

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Post-COVID syndrome is associated with increased extracellular purine bases and neutrophil extracellular traps in the blood plasma

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ABSTRACT

Post-COVID syndrome is characterized by fatigue, reduced exercise tolerance, muscle and joint pain, and psycho-emotional disorders. In the development of a generalized body response in a viral infection, abnormal defense responses are of great importance. We studied neutrophils, neutrophil extracellular traps (NETs), DNA degradation products (purine nitrogenous bases, PNBs), and traditional biochemical parameters.

Aim. To determine biochemical parameters and the number of NETs and PNBs in the peripheral blood of patients with post-COVID syndrome.

Materials and methods. The study included outpatients (n = 21) aged 18–59 years (36 [27 ÷ 50]). The control group consisted of 20 individuals aged 18–59 years (38.5 [29 ÷ 51.5]) without a past medical history of the coronavirus infection. All patients underwent a physical examination, their medical history was assessed, and the level of NETs and PNBs in the venous blood was determined.

Results. 11 patients had a mild form of the disease in their past medical history, 7 – moderate, and 3 – severe. The most common symptoms in the patients were fatigue, headache, epigastric pain, dizziness, and joint pain. Hair loss and dyspnea were less common. The concentration of NETs and PNBs was higher in the patients with post-COVID syndrome than in the control group ($p < 0.05$). We detected NETs in the patients with post-COVID syndrome only in the form of filamentous structures. The concentration of extracellular purine bases in the blood of the patients with post-COVID syndrome was the highest in patients with moderate and severe acute periods. In patients with a mild acute period, the concentration of PNBs was 7.38 [0.0 ÷ 60.7] mg / ml, and in patients with moderate and severe acute periods – 19.15 [0.0 ÷ 33.5] and 34.19 [3.35 ÷ 70.0] mg / ml, respectively.

Conclusion. Extracellular purine bases in concentrations capable of causing secondary alteration of cells are found in the peripheral blood of patients with post-COVID syndrome. Post-COVID syndrome is accompanied by the formation of filamentous NETs in the blood of patients.

Keywords: post-COVID syndrome, extracellular purine bases, neutrophil extracellular traps, COVID-19, pathogenesis

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Постковидный синдром ассоциирован с повышением внеклеточных пуриновых оснований и нейтрофильных экстраклеточных ловушек в плазме крови

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РЕЗЮМЕ

Постковидный синдром характеризуется высокой утомляемостью, снижением толерантности к физической нагрузке, болями в мышцах и суставах, наличием психоэмоциональных проблем. В развитии генерализованной реакции организма при вирусном инфицировании большое значение имеют аномальные реакции защитных систем. Мы исследовали нейтрофилы и формируемые ими экстраклеточные ловушки (НЭЛ) совместно с продуктами деградации волокон ДНК (пуриновые азотистые основания, ПАО), а также традиционные клинико-лабораторные показатели.

Цель. Определение ряда лабораторных показателей, а также количества НЭЛ и уровня ПАО в периферической крови больных с постковидным синдромом.

Материалы и методы. В исследование включены амбулаторные пациенты (n = 21) в возрасте 18–59 лет (36 [27÷50]). Группу сравнения составили 20 лиц в возрасте 18–59 лет (38,5 [29÷51,5]) без перенесенной коронавирусной инфекции. Всем пациентам проводились сбор жалоб, оценка анамнеза, физикальный осмотр, определение НЭЛ и ПАО в венозной крови.

Результаты. Легкое течение заболевания в анамнезе имелось у 11, среднетяжелое – у 7, тяжелое – у 3 пациентов. Наиболее частыми симптомами в нашей группе обследованных пациентов были слабость, головная боль, боль в эпигастрии, головокружение, боль в суставах. Более редкими симптомами являлись выпадение волос и одышка. Концентрация НЭЛ и ПАО была выше в основной группе, чем в группе сравнения ($p < 0,05$). Мы выявляли НЭЛ у больных с постковидным синдромом только в нитевидной форме. Концентрация внеклеточных пуриновых азотистых оснований в плазме крови больных с постковидным синдромом была наиболее высокой у больных со среднетяжелым и тяжелым течением острого периода. У больных, перенесших острый период заболевания в легкой форме, концентрация ПАО составляет 7,38 [0,0÷60,7] мг/мл, а у больных со среднетяжелой и тяжелой формой острого периода – 19,15 [0,0÷33,5] и 34,19 [3,35÷70,0] мг/мл соответственно.

Заключение. В периферической крови больных с посткоронавирусным синдромом обнаруживаются внеклеточные ПАО в концентрации, способной вызвать вторичную альтерацию клеток. Постковидный синдром сопровождался формированием в периферической крови больных НЭЛ в нитевидной форме.

Ключевые слова: постковидный синдром, внеклеточные пуриновые азотистые основания, нейтрофильные экстраклеточные ловушки, ковид-19, патогенез

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

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

Соответствие принципам этики. Исследование одобрено этическим комитетот РНИМУ им. Н.И. Пирогова (протокол № 203 от 21.12.2021).

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INTRODUCTION

Post-COVID syndrome is a complex of symptoms that occurs in 20% of people who have had the coronavirus infection (COVID-19). Patients with post-COVID syndrome suffer from headaches, asthenia, arthralgia, myalgia, consequences of organ dysfunction (lungs, heart, gastrointestinal tract (GIT), skin, kidneys, liver etc.), and neuropsychiatric disorders. Pathological manifestations of post-COVID syndrome persist for three or more months after the coronavirus infection. The syndrome is diagnosed clinically based on a past infectious medical history. Some researchers differentiate the concepts of "post-COVID" and "long COVID", believing that post-COVID is a complication of the cured COVID-19, while long COVID is chronic persistence of the virus in the body. The pathogenesis of post-COVID syndrome is unclear and poorly understood. The etiology of post-COVID syndrome is directly related to the past coronavirus infection. It is assumed that residual inflammation, prolonged persistence of SARS-CoV-2 in latent foci, the formation of autoantibodies, and even social isolation can be significant in the pathogenesis of post-COVID syndrome.

At the same time, there are some reasons to believe that the manifestations of the post-COVID syndrome are associated with an increased concentration of extracellular purine nitrogenous bases (PNBs) and formation of neutrophil extracellular traps (NETs), leading to the development of immune thrombotic disorders.

The aim of the study was to compare clinical and laboratory parameters and the number of NETs and extracellular PNBs in the peripheral blood of patients with post-COVID syndrome, depending on the severity of the coronavirus infection.

MATERIALS AND METHODS

An open, comparative study included 21 patients aged 18 to 59 years (36 [27 ÷ 50]). Inclusion criteria: men and women aged 18–59 years with the confirmed diagnosis of COVID-19 in their past medical history; severe, moderate or mild forms of the coronavirus infection; not earlier than 1 month but not later than 12 weeks (3 months) from the onset of the first symptoms of COVID-19. Exclusion criteria: age over 60 years old; extremely severe course of COVID-19; the presence of chronic diseases incompatible with life or life expectancy of less than a year; asymptomatic carriers of the coronavirus infection (or those who contacted with COVID-19 patients but did not get ill).

The control group consisted of 20 individuals aged 18–59 years (38.5 [29 ÷ 51.5]) without a past medical history of the coronavirus infection. These patients went to the physician of the outpatient clinic for a regular check-up.

All patients underwent a physical examination, their medical history was taken, and anamnestic data were assessed. A past medical history of COVID-19 was confirmed by a positive test to detect SARS-CoV-2 RNA using nucleic acid amplification techniques or SARS-CoV-2 antigen using immunochromatography regardless of clinical manifestations or after patients with clinically confirmed COVID-19 were tested positive for immunoglobulin (Ig) A, IgM, and/or IgG antibodies. Standard criteria were used to assess the severity of disease manifestations.

A mild form of the infection was characterized by a body temperature lower than 38°C, cough, weakness, and sore throat. A moderate form of infection was characterized by a body temperature of 38°C or higher, respiratory rate higher than 22 breaths / min, dyspnea on exertion, pulmonary changes on chest computed tomography (CT) or X-ray typical of a viral infection (the volume of the lesion is minimal or moderate; stage 1–2 according to CT findings), oxygen saturation (SpO_2) of less than 95%, and an increase in the level of C-reactive protein above 10 mg / l. A severe form of infection was characterized by respiratory rate higher than 30 breaths / min, $SpO_2 \leq 93\%$, $PaO_2 / FiO_2 \leq 300$ mmHg, and hemodynamic instability (systolic blood pressure lower than 90 mmHg or diastolic blood pressure lower than 60 mmHg, diuresis less than 20 ml / hour). The volume of lung damage is significant or subtotal (stage 3–4 according to CT findings). Data on the disease severity were obtained from patients' medical histories, epicrises, and health records.

To determine the blood biochemical parameters of COVID-19 patients and patients of the control group, an automatic clinical chemistry analyzer Olympus 5800 (JP, Olympus Corporation, USA) was used. Standard methods using manufacturer's reagents were employed to carry out tests in the laboratory of the City Government-Funded Healthcare Institution "Diagnostic Clinical Center No. 1 of the Moscow City Health Department". The study was approved by the protocol No. 203 at a meeting of the Ethics Committee at Pirogov Russian National Research Medical University on December 21, 2021. Each patient signed an informed consent to participate in the study. The study was conducted at the City Government-Funded Healthcare Institution "Diagnostic Clinical Center No.

1 of the Moscow City Health Department". The Department of Polyclinic Therapy and the Department of Pathophysiology and Clinical Pathophysiology of Pirogov Russian National Research Medical University were used as the clinical site.

In addition to standard clinical laboratory studies, two new laboratory methods were used in the study: determination of the NET concentration and the PNB level in the blood plasma.

Determination of the number of neutrophil extracellular traps

Cell fractionation. Cell fractions of neutrophils were used in the study. Venous blood (10 ml) of patients was placed in a siliconized tube containing EDTA to prevent clotting. To isolate neutrophils from EDTA-treated venous blood, the blood was diluted two-fold with sodium phosphate buffer solution (pH 7.4) and layered on a double Ficoll – Verografin density gradient. The density of the gradient upper layer was 1.077, the density of the bottom layer was 1.190. Centrifugation (1,600 rpm, 30 min) resulted in a ring of granulocytes with purity of 98–100% at the boundary between the gradients. Erythrocytes were deposited on the bottom of the tube. The ring of neutrophil granulocytes was collected, placed into centrifuge tubes, and washed twice with a buffer solution to remove Ficoll. Centrifugation was used to pellet the cells (1,200 rpm, 15 min). Sterilely isolated neutrophils were transferred to the RPMI-1640 medium and then used in cell culture experiments. The viability of the isolated neutrophils was at least 95%, which was determined in the test with 0.1% Trypan Blue solution.

Immunofluorescent staining of neutrophil extracellular traps. Fluorescence microscopy was used to detect and quantify NETs. The technique is proprietary and is described in detail in the RF Patent Application No. 2021104936. The results were expressed in percentage as the ratio of the number of NETs to the total number of neutrophils. The fluorescent dye Syber Green (Evrogen, Russian Federation) which specifically binds to double-stranded DNA was used to detect NETs.

Detection of purine nitrogenous bases

The method is based on the interaction between PNBs and silver nitrate to form a colored compound. The blood plasma was subjected to high-speed centrifugation at 20,000 rpm for 30 min and stored at –26°C. Chloroform was used to extract PNBs from the blood plasma. To do this, 2 ml of chloroform was added to 0.5 ml of the blood plasma, and this mixture was processed on a vibration platform at room temperature for 1 hour. Chloroform (1 ml) with PNBs dissolved in it was collected, and the samples were dried in a vacuum evaporator. The dry precipitate containing PNBs was dissolved in 3 ml of 10% NaOH solution, and 500 µl of 5% $AgNO_3$ solution prepared in 10% aqueous ammonia was added. The reaction resulted in a light brown staining. The samples were photometered at a wavelength of 610 nm. Adenine (Sigma, USA) was used to construct a calibration curve. The calibration curve was linear in the range of 0–10 mg / ml.

Statistical processing of the data was performed using STATISTICA 12.0 (StatSoft). Descriptive statistics were presented as continuous quantitative data: as the mean and the standard error of the mean ($M \pm m$) in normal distribution and as the median and the interquartile range (*Me* [25÷75]) in non-normal distribution . Comparison of quantitative variables was performed using the Mann – Whitney U test and Kruskal – Wallis one-way analysis of variance. The difference was considered statistically significant at $p < 0.05$.

RESULTS

Weakness, headache, epigastric pain, dizziness, and joint pain were the most common symptoms in patients with post-COVID syndrome. Hair loss and dyspnea were less common. A similar study named weakness, myalgia, headaches, and vegetative symptoms as the most common symptoms in patients with post-COVID syndrome [1]. The authors suggest that these symptoms are more typical of post-COVID syndrome, that they do not require specific treatment and may be associated with microangiopathy and endothelial dysfunction.

The group of patients with post-COVID syndrome demonstrated the activity of alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP), increased by 1.7, 2.1, and 3.7 times, respectively. These changes indicate the development of hepatotoxicity, but do not allow for a complete understanding of the post-COVID syndrome pathogenesis.

The most significant changes in patients with post-COVID syndrome were detected when studying NETs and PNBs levels in the blood plasma.

The study of NETs in patients with post-COVID syndrome shows that they are formed as thin single filaments of considerable length. The dimensions of DNA strands exceed several tens of cell diameters.

The peculiarity of NETs in patients with post-COVID syndrome is that DNA fibers do not form a network structure and are not capable of capturing with subsequent retraction of apoptotic cells (Fig. 1–3).

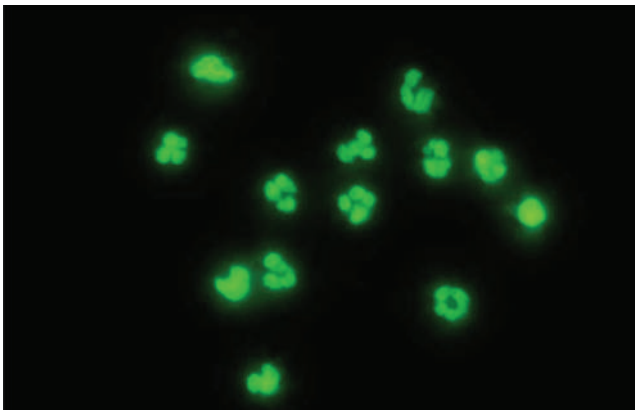

Fig. 1. Intact neutrophils of healthy donors

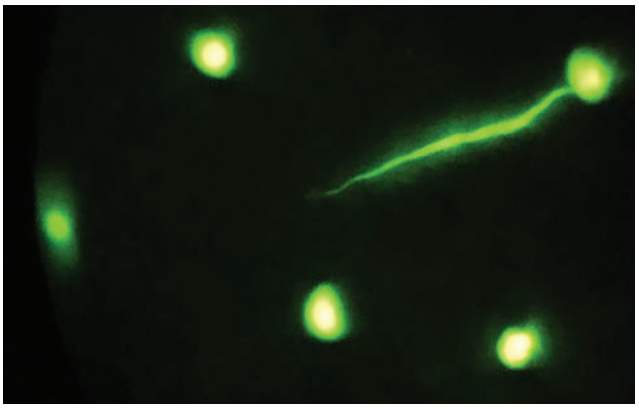

Fig. 2. Initial stages of NET formation in post-COVID syndrome. Ejection of a single strand of nuclear DNA from the cell nucleus; neutrophil incubation is 30 min

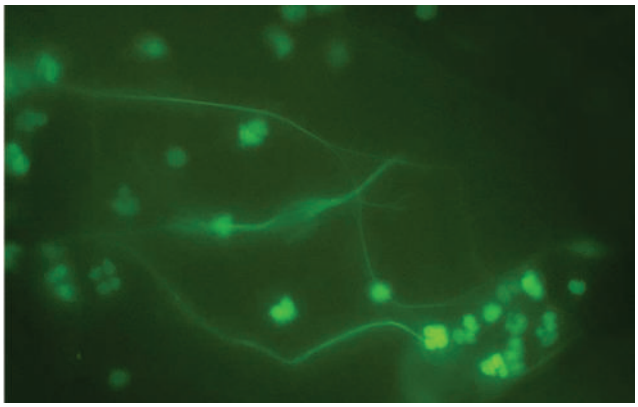

Fig. 3. NETs in post-COVID syndrome; neutrophil incubation is 4 hours

In our previous studies, we found that the morphological structure of NETs depends on the type of inflammation. The formation of NETs in the form of single strands of nuclear DNA (Fig. 2, 3) indicates sterile inflammation in patients.

The number of NETs in patients with post-COVID syndrome is insignificant, but they are constantly reproduced over a long period of time (3 months and longer). The results of determining the number of NETs in patients with post-COVID syndrome, depending on the severity of the disease, are presented in the table.

Our studies showed that the number of NETs in the post-COVID period was on average 2.6 times greater in patients who had a moderate form of the coronavirus infection, compared with patients with a mild form of the disease. At the same time, NETs were not detected at all in patients with a severe form of the disease in the past (Table).

Table

Neutrophil extracellular traps and extracellular purine nitrogenous bases in patients with post-COVID syndrome who have experienced an acute period of the disease in mild, moderate, and severe forms, *Me* [25÷75], *M* ± *m*

Parameter	Control group, *n = 20*	Patients with post-COVID syndrome, *n* = 21		
		Mild form, *n* =11	Moderate form, *n* = 7	Severe form, *n* = 3
NETs, %	0.00	0.00 [0.00÷1.31]* 0.61 ± 0.23	0.00 [0.00÷0.56]* 1.61±1.52	0.00
Extracellular PNBs, mg / ml	0.00	7.38 [0.0÷60.7]* 23.27 ± 8.9	19.15 [0.0÷33.5]* 22.89 ± 8.36	34.19 [3.35÷70.0]* 35.84 ± 19.25

* $p < 0.05$ compared with the control group (according to the Kruskal – Wallis analysis of variance).

We suggest that the formation of filamentous NETs in patients with post-COVID syndrome is one of the reasons for a significant increase in the concentration of PNBs in the blood. Thin strands of DNA are destroyed by the effect of DNases localized on chromatin. An increase in the activity of nuclear DNases occurs during chromatin despiralization, which is observed in the formation of NET networks. The interaction of DNases with decondensed chromatin leads to the accumulation of nucleotides in the intercellular space. Ectoenzymes CD39 and CD73 localized on the surface of blood cells and endotheliocytes catalyze

hydrolysis of nucleotides to nitrogenous bases and can cause an increase in the concentration of PNBs. Tests studying the blood serum of patients with COVID-19 showed increased levels of extracellular DNA and specific NET markers [2].

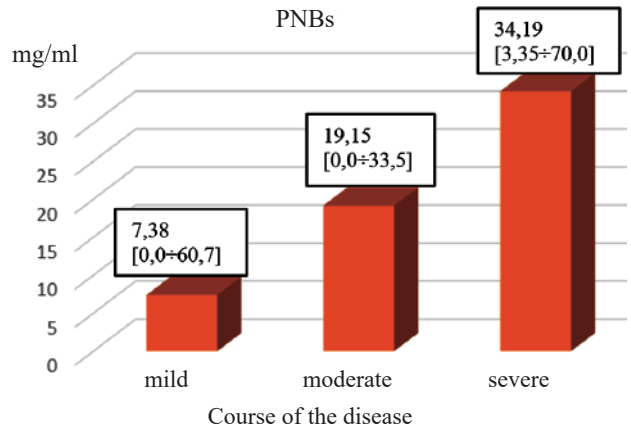

Fig. 4. Extracellular purine nitrogenous bases (PNBs) in patients with post-COVID syndrome who have had the disease in mild, moderate, and severe forms, mg / ml, *Me* [25÷75]

The data obtained show that the activity of enzymes catalyzing the hydrolysis of extracellular nucleotides to free nitrogenous bases is increased on the surface of patients' blood cells [3]. The results demonstrate that the concentration of extracellular PNBs in the blood of patients with post-COVID syndrome was elevated in proportion to the severity of the disease (Table, Fig. 4).

DISCUSSION

Molecular inducers of NET formation are currently unknown. However, it has been established that this process is accompanied and possibly initiated by the components of pathogenic microorganisms, activated platelets, complement system proteins, autoantibodies, and proinflammatory cytokines. NETs take part in thrombosis and occlusion of small vessels through activation of the contact pathway of blood coagulation, electrostatic interactions between histone proteins and platelet phospholipids, destruction of antithrombin III by neutrophil elastases, and induction of interleukin (IL) 1β synthesis. Activation of IL1β and platelets leads to an increase in the NET formation, and an increase in the concentration of its components (extracellular DNA, myeloperoxidase (MPO) – DNA complex, citrullinated histone H3) in the blood serum of COVID-19 patients correlates with the severity of the disease and the development of thrombotic disorders [2,4,5].

In the present study, we detected NETs in patients with post-COVID syndrome only in the form of filamentous structures. No other morphological variants of NETs were found in the examined patients. Moreover, NETs were absent in patients with a severe course of the disease. At the same time, the PNB concentration in patients with post-COVID syndrome was increased in proportion to the severity of the disease. Patients who had had a severe form of the coronavirus infection did not have NETs in the post-COVID period, but we assume that filamentous networks of DNA fibers are produced by other blood cells. Therefore, an increase in the PNB concentration in patients with post-COVID syndrome in proportion to an increase in the disease severity seems to be quite logical and uncontroversial.

Extracellular PNBs are toxic to the body, but the mechanism of their damaging effect is unknown. Animal models with chronic adenine diet showed rapid progression of kidney disease with extensive tubulointerstitial fibrosis, tubular atrophy, crystallization, pronounced vascular calcification, and cardiovascular disorders [6–9].

Myocardial ischemia significantly increases the activity of CD39 and CD73 ectoenzymes localized on the surface of platelets, catalyzing extracellular hydrolysis of adenyl nucleotides to adenine, in patients after myocardial infarction. As a result, the levels of extracellular adenine and troponin increase in such patients [10].

We observed an increase in the concentration of extracellular PNBs in patients with post-COVID syndrome (more than 3 months) and suggest that they are an endogenous damaging factor in the pathogenesis of the post-COVID period (secondary alterations), especially in patients with drowsiness, joint pain, headache, weakness, and hair loss. We believe that asthenic syndrome, which persisted in more than half of patients in the post-COVID period, is based on persistent and prolonged increased production of extracellular PNBs, which act as a damaging factor. The results of our study make it relevant to clarify the causes of a significant long-term increase in the concentration of extracellular PNBs in post-COVID syndrome.

CONCLUSION

Extracellular PNBs found in the peripheral blood of patients with post-COVID syndrome can cause secondary cell alterations.

Post-COVID syndrome is accompanied by the formation of filamentous NETs in the peripheral blood.

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Interferons alpha and gamma, pidotimod, and tilorone in the treatment of acute respiratory infections in patients with allergic rhinitis: a prospective, cohort clinical and immunological study

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ABSTRACT

Aim. To compare the clinical efficacy and influence on interferon (IFN) production / sensing of drugs with immune-mediated antiviral effects, which potentiate type 1 (T1) immune responses, in the treatment of acute respiratory infections (ARI) in patients with allergic rhinitis.

Materials and methods. 146 ARI patients with remission of seasonal allergic rhinitis were divided into 4 cohorts. In addition to symptomatic therapy, patients received either 2,000 IU of IFNγ in each nasal passage 5 times a day; or rectal suppositories containing 10^6 IU of IFN-α2b and antioxidants (AO) twice a day; or gel with IFN-α2b and AO intranasally 3 times a day; or 400 mg of pidotimod *per os* twice a day; or 125 mg of tilorone *per os* on days 1, 2, 4, and 6. The severity of ARI was determined daily as the sum of 10-point scores for 15 symptoms. Serum concentrations of IFNα and IFNγ and the ability of blood cells to produce these cytokines *ex vivo* spontaneously and upon stimulation with Newcastle disease or phytohemagglutinin were studied using enzyme-linked immunosorbent assay (ELISA). The proportions of circulating lymphocytes expressing type I IFN receptor subunit 2 (CD118) or IFNγ receptor α-chain (CD119) were determined by flow cytometry.

Results. ARI symptoms in all cohorts generally regressed in a similar way. However, from day five of the treatment, pidotimod relieved symptoms more effectively than other drugs. In patients treated with tilorone, the regression of ARI manifestations was delayed in the first two to three days, followed by rapid symptom reduction. An initial decrease in the induced production of IFNγ was found in patients treated with pidotimod, and a tendency to a decrease in this parameter was noted in other cohorts. The induced production of IFNγ after the treatment in all groups did not differ from that in healthy donors. No significant changes and differences in the proportions of $CD118^+$ and $CD119^+$ lymphocytes were found between the cohorts, except for a decrease in the number of $CD118^+$ cells after the treatment with tilorone. In patients treated with IFN-α2b + AO, the proportions of $CD119^+$ and $CD118^+$ lymphocytes tended to increase slightly.

Conclusion. Drugs polarizing immune responses toward the Th1 type are a useful option for treating ARI in patients with allergic rhinitis.

Keywords: acute respiratory infections, interferon gamma, interferon alpha-2b, antioxidants, pidotimod, tilorone, interferon receptors, type 1 immune responses

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Интерфероны альфа и гамма, пидотимод и тилорон в лечении острых респираторных инфекций у пациентов с аллергическим ринитом: проспективное когортное клинико-иммунологическое исследование

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РЕЗЮМЕ

Цель – сравнить клиническую эффективность и влияние на выработку и рецепцию интерферонов (ИФН) препаратов с иммуноопосредованным противовирусным действием, потенцирующих иммунный ответ 1-го типа (Т1), в лечении острых респираторных инфекций (ОРИ) у пациентов с аллергическим ринитом.

Материалы и методы. Больные ОРИ (n = 146) с сезонным аллергическим ринитом в стадии ремиссии распределены на четыре когорты. Помимо симптоматической терапии пациенты получали либо 2 000 МЕ ИФН-γ в каждый носовой ход 5 раз/сут; либо ректальные свечи, содержащие 10^6 МЕ ИФН-α2b и антиоксиданты (АО), 2 раза/сут гель с ИФН-α2b и АО интраназально 3 раза/сут; либо 400 мг пидотимода *per os* 2 раза/сут; либо 125 мг тилорона *per os* в 1, 2, 4 и 6-е сут. Выраженность клинических проявлений ОРИ определяли ежедневно по сумме 10-балльных оценок 15 симптомов. Концентрации ИФН-α и ИФН-γ в сыворотке крови и способность клеток крови вырабатывать эти цитокины *ex vivo* спонтанно и при стимуляции вирусом болезни Ньюкасла или фитогемагглютинином изучали с помощью иммуноферментного анализа. Доли циркулирующих лимфоцитов, экспрессирующих субъединицу-2 рецептора ИФН I типа (CD118) или α-цепь рецептора ИФН-γ (CD119), определяли методом проточной цитофлуориметрии.

Результаты. Симптомы ОРИ во всех когортах регрессировали в целом сходным образом. Однако пидотимод с 5-х сут лечения купировал симптомы эффективнее других препаратов, а на фоне приема тилорона регрессия проявлений ОРИ задерживалась в первые 2–3 сут, после чего симптомы быстро угасали. Обнаружено исходное снижение индуцированной продукции ИФН-γ у пациентов, подлежащих лечению пидотимодом, и тенденция к уменьшению этого показателя в других когортах. После лечения индуцированная выработка ИФН-γ во всех группах не отличалась от таковой у здоровых доноров. Не установлено существенной динамики и отличий между группами по долям $CD118^+$- и $CD119^+$-лимфоцитов, за исключением снижения количества $CD118^+$-клеток на фоне приема тилорона. Лечение ИФН-α2b с АО вызывало незначительную тенденцию к увеличению доли $CD119^+$- и $CD118^+$-лимфоцитов.

Заключение. Препараты, поляризующие иммунный ответ в направлении Т2→Т1, являются полезной опцией в лечении ОРИ у больных с аллергическим ринитом.

Ключевые слова: острые респираторные инфекции, интерферон гамма, интерферон альфа-2b, антиоксиданты, пидотимод, тилорон, рецепторы интерферонов, иммунный ответ 1-го типа

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

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

Соответствие принципам этики. Исследование одобрено локальным этическим комитетом ЦНИИ эпидемиологии (протокол № 79 от 15.12.2017).

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INTRODUCTION

Treatment of acute respiratory viral infections (ARVI) remains largely the focus of discussions among scientists and doctors. The vast majority of used and studied etiotropic and pathogenetic drugs are far from the gold standard in this regard, especially in case of ARVI therapy in patients with concomitant allergic respiratory diseases. Allergic diseases and respiratory infections have a number of common and / or mutually potentiating links in their pathogenesis. Histamine, leukotrienes, prostaglandins, and many cytokines and chemokines are mediators of both allergic and infectious inflammation [1–5].

The development of allergic rhinitis and the most common phenotypes of other allergic respiratory diseases is associated with the predominance of type 2 (T2) immune response [6, 7]. At the same time, human rhinovirus, respiratory syncytial virus (RSV), and some other pathogens of ARVI polarize immune responses toward the Th2 type [8, 9]. Human rhinoviruses species A and B use the intercellular adhesion molecule 1 (ICAM-1) to enter the cell [10], and an increase in the expression of this molecule is an important link in the pathogenesis of respiratory allergic diseases [11, 12]. In addition, rhinoviruses themselves enhance the expression of ICAM-1 [13]. This does not include all the mechanisms by which respiratory viruses can provoke or aggravate allergic inflammation, and allergic respiratory disease can facilitate infection and create conditions for a more severe or atypical course of ARVI [14]. Patients with remission of allergic rhinitis retain minimal signs of persistent T2 inflammation, which makes them more sensitive to non-specific irritants [15] and *a priori* should affect the course of ARVI.

The aim of this study was to compare the clinical efficacy and effect on the production and sensing of type I and type II IFNs of several immunostimulants and drugs with immune-mediated antiviral effects, which are approved for clinical use in the Russian Federation for the treatment of acute respiratory infections (ARI) in patients with remission of concomitant allergic rhinitis.

Drugs were chosen as objects of this study due to their proven or implied ability to switch the balance of the prevailing immune response: T2→T1. Interferon-γ (IFN-γ) in nasal dosage forms was chosen as a key mediator of T1 immune responses and a stimulator of cellular antiviral defense [16]. IFN-α2b in topical and rectal dosage forms with added antioxidants was considered not only as the most important factor of antiviral innate immunity, but also as a molecule that potentiates the production of IFN-γ and suppresses the production of T2 cytokines [17, 18].

Pidotimod has proven to be effective in the prevention of respiratory infections [19], including patients with allergic respiratory diseases [20]. This synthetic dipeptide stimulated T cell immune responses, suppressed T2 inflammation, and increased the IFN-γ / interleukin (IL)-4 ratio in the serum of patients with respiratory allergies [21, 22].

The ability of tilorone to induce the production of type I IFNs has been known for more than 50 years [23]. Recently, it has been shown that this drug enhances (modulates) the production of IFN-γ, IFN-λ, and some other cytokines in intact animals and under experimental conditions of influenza *in vivo* [24, 25] and induces polarization of the immune response toward the T1 type [26].

MATERIALS AND METHODS

147 patients with ARI and remission of concomitant seasonal allergic rhinitis were followed up in City Polyclinic No. 180 of Moscow Healthcare Department, Scientific Advisory Clinical Diagnostic Center, and Clinical Department of Infectious Pathology of Central Research Institute of Epidemiology of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing

during the autumn – winter epidemic seasons of 2016–2019. The study was carried out in accordance with the requirements of the World Medical Association (WMA) Declaration of Helsinki "Ethical principles for medical research involving human subjects" as amended by the 52nd WMA General Assembly (2000) and "Rules of Clinical Practice in the Russian Federation" approved by the order of the Ministry of Health of the Russian Federation No. 266 dated June 19, 2003.

Inclusion criteria: clinical presentation of ARI within 48 hours after the onset of the first symptoms; history of seasonal allergic rhinitis verified at least 2 years ago; remission of allergic rhinitis; the level of immunoglobulins E (IgE) in the blood serum ≥ 100 IU / ml; age from 18 to 65 years; a voluntary informed consent to participate in scientific research.

Exclusion criteria: treatment with antiviral and immunomodulatory drugs within 1 month before therapy; the presence of ARI complications at the time of the initial visit; autoimmune diseases; chronic diseases of the cardiovascular system, gastrointestinal tract, and endocrine system, requiring medication intake during the study period; chronic obstructive pulmonary disease; tuberculosis; HIV infection; drug addiction; hypersensitivity to the components of drugs studied.

Withdrawal criteria: an allergic or other adverse reaction to any drugs manifested during the study; non-compliance with the frequency and dosage regimen; refusal of the patient to continue participating in the study.

Patients included in the study were divided into 4 cohorts (Table 1).

Table 1

Characteristics of the patients included in the study

Parameter		Patient groups				Statistically significant differences
Group number		1	2	3	4	
Treatment		IFN-γ	IFN-α + AO	Pidotimod	Tilorone	–
Number of patients		60	27	28	31	
Age, years, *Me* (Q_1–Q_3; *Min–Max*)		29 (26–38; 19–58)	46 (40–50; 27–62)	44 (40–50; 27–62)	29 (27–39; 18–57)	*
Sex	Men, *n* (%)	36 (60)	15 (56)	14 (50)	12 (39)	–
	Women, *n* (%)	24 (40)	12 (44)	14 (50)	19 (61)	
Erythrocyte sedimentation rate, mm / h, *Iu* (Q_1–Q_3; *Min–Max*)		6 (5–12; 2–25)	6 (4–9; 2–40)	10 (4–14; 1–24)	7 (2–5; 1–29)	–
Number of leukocytes in blood, ×10^3/mcl, Me (Q_1–Q_3; *Min–Max*)		6.7 (4.9–9.1; 3.1–13.1)	6.4 (5–7.7; 3.4–13.9)	6.5 (5.8–8.1; 3.1–12.8)	7.1 (5.7–9.8; 4.3–16.7)	–
The proportion of lymphocytes among blood leukocytes, %, *Me* (Q_1–Q_3; *Min–Max*)		36 (30–40; 11–48)	37 (30–44; 17–64)	36 (29–38; 14–64)	36 (30–40; 11–48)	–
Serum IgE concentration, IU / ml, *Me* (Q_1–Q_3; *Min–Max*)		297 (173–450; 102–822)	231 (159–417; 101–621)	218 (180–304; 100–598)	230 (185–394; 105–725)	–
Number of patients with identified ARI viruses, *n* (%)	Rhinoviruses	15 (25)	7 (26)	7 (25)	6 (19)	–
	Influenza A virus	19 (32)	10 (37)	9 (32)	10 (32)	–
	Influenza B virus	0	0	0	1 (3)	–
	Human parainfluenza viruses 1–4	4 (7)	1 (4)	1 (4)	1 (3)	–
	RSV	4 (7)	2 (7)	2 (7)	2 (6)	–
	Adenoviruses	5 (8)	2 (7)	1 (4)	4 (13)	–
	Coronaviruses	0	0	0	1 (1)	–
	Not identified	13 (22)	6 (22)	8 (29)	6 (19)	–

Note: RSV – respiratory syncytial virus.

* $p_{1,2,3,4} < 0.001$ (the Kruskal – Wallis test); $p_{1\text{-}2} < 0.001$, $p_{1\text{-}3} < 0.001$, $p_{2\text{-}4} < 0.001$, $p_{3\text{-}8} < 0.001$ (the Dunn's test).

In cohort 1 (IFN-γ group), the patients were treated with 2,000 IU of human recombinant IFN-γ (Pharmaclon, Russian Federation) in 2 drops of aqueous solution into each nasal passage 5 times a day for 7 days, which was a part of a complex therapy. In cohort 2 (IFN-α + antioxidants (AO) group), the patients received rectal suppositories containing 1 million IU of IFN-α2b, 0.055 g of alpha-tocopherol acetate, and 0.0081 g of ascorbic acid (Feron, Russian Federation) twice a day and a strip of gel 0.4–0.5 cm long, 1 gram of which contained 36,000 IU of IFN-α2b, 0.055 g of α-tocopherol acetate, 0.00128 g of benzoic acid, and 0.001 g of citric acid monohydrate (Feron, Russian Federation), 3 times a day in each nasal passage for 7 days. In cohort 3 (pidotimod group), the patients received 400 mg of pidotimod (Doppel Farmaceutici Srl, Italy) *per os* 2 times a day for 10 days. In cohort 4 (tilorone group), the patients received 125 mg of tilorone (Nizhpharm, Russian Federation) *per os* on days 1, 2, 4, and 6. In addition to these drugs, the patients received symptomatic treatment (irrigation procedures, decongestants, paracetamol at temperatures above 38.5°C).

All patients underwent a comprehensive examination, including history taking, physical examination, complete blood count, determination of the IgE level in the blood serum, verification of pathogens of respiratory infection, analysis of the interferon system and their receptors, and if necessary, clinical investigations (computed tomography of the sinuses, chest X-ray, electrocardiogram). Blood and nasopharyngeal samples for laboratory studies were taken in the first 48 hours from the onset of the disease before treatment and on day 7 of the treatment. All patients were followed up until complete recovery.

The main criterion (primary endpoint) for comparing the effectiveness of different treatment options was the influence of ARI clinical manifestations on regression. The overall severity of ARI was determined as the sum of 10-point scores for each of the symptoms listed below. Hyperthermia was assessed as follows: 1 point for body temperature ≥ 37°C; another 1 point for each additional temperature rise by 0.2°C; 10 points for any temperature ≥ 38.8°C. Other 14 symptoms and signs were assessed as follows: weakness, decreased appetite, nasal congestion, nasal discharge, itchy nose, itchy throat, sore throat, hoarseness, cough, sneezing, myalgia, headache, chest pain, and pain in the eyes. The results of the assessment of ARI clinical manifestations were recorded daily for 7 days in a specially developed individual diary, in which patients also had to register possible adverse events.

The pathogens of ARI in a nasopharyngeal swab were identified by the polymerase chain reaction using the AmpliSens ARVI-screen-FL and AmpliSens Influenza virus A/B-FL diagnostic test systems (Central Research Institute of Epidemiology, Russian Federation). Complete blood count was performed using a hematology analyzer (Beckman Coulter, USA) with determination of a standard set of parameters.

The ability of blood cells to produce IFN-α and IFN-γ *ex vivo* upon stimulation by the Kansas strain of the Newcastle disease virus (NDV) or phytohemagglutinin (PHA) (PanEco, Russian Federation), respectively, and without these stimuli was evaluated using the method proposed by S.S. Grigoryan et al. [27], as described earlier [28]. The levels of IFN-α and IFN-γ in the cell culture supernatant and blood serum were determined by ELISA using eBioscience kits (USA) and a fully-automated microplate reader Anthos 2020 (Anthos Labtec Instruments GmbH, Austria) at a wavelength of 450 nm with a correction at 620 nm.

The proportion of circulating lymphocytes expressing IFN-α / β receptor subunit 2 (CD118) and IFN-γ receptor α-chain (CD119) in peripheral blood was determined by flow cytometry on the EPICS XL cytometer (Beckman Coulter, USA) using PE-conjugated antibody to CD118 (Beckman Coulter, USA), PE-conjugated antibody to CD119 (eBioscience, USA), and BD FACS lysis solution (Becton Dickinson, USA) as described earlier [29]. Data from the analysis of biological samples collected from 30 healthy individuals comparable in gender and age with the studied population were used as a conditional norm of laboratory parameters.

A statistical analysis was carried out using the Statistica 18 software (StatSoft Inc., USA). Paired comparisons of independent and dependent samples in terms of quantitative characteristics were performed using the Mann – Whitney and Wilcoxon tests, respectively. The Kruskal – Wallis and Dunn's tests were used for multiple comparison of independent samples in terms of quantitative indicators. Independent groups were compared by nominal characteristics using the χ^2 test. All quantitative data

in the tables and in the figure are presented as *Me* (Q_1–Q_3; *Min–Max*), where *Me* is the median, Q_1 is the lower quartile, Q_3 is the upper quartile; *Min* is the minimum, *Max* is the maximum. The differences were considered statistically significant at $p < 0.05$. At $0.05 \leq p < 0.1$, a trend was claimed.

RESULTS

The cohorts of patients did not differ in the etiology of ARI, duration of infection during treatment, serum IgE level, erythrocyte sedimentation rate, and number of leukocytes and lymphocytes in peripheral blood. The groups were generally comparable in gender: a slight predominance of women in the tilorone cohort did not lead to statistically significant gender differences between the groups (Table 1). Sensitization to birch pollen allergens as a cause of seasonal allergic rhinitis, which was in remission during the study period, dominated in all cohorts. At the same time, patients in the IFN-α + AO and pidotimod groups were older than those in the IFN-γ and tilorone cohorts. Intergroup heterogeneity was also noted in terms of the initial severity of clinical manifestations of ARI. This was associated with a higher score for key symptoms of the disease in the tilorone cohort compared with patients who were prescribed IFN-γ and pidotimod (Fig. 1). The higher score in the tilorone group was mainly due to a high degree of myalgia, headache, decreased appetite, weakness, and eye pain. The groups did not differ significantly with respect to other ARI symptoms.

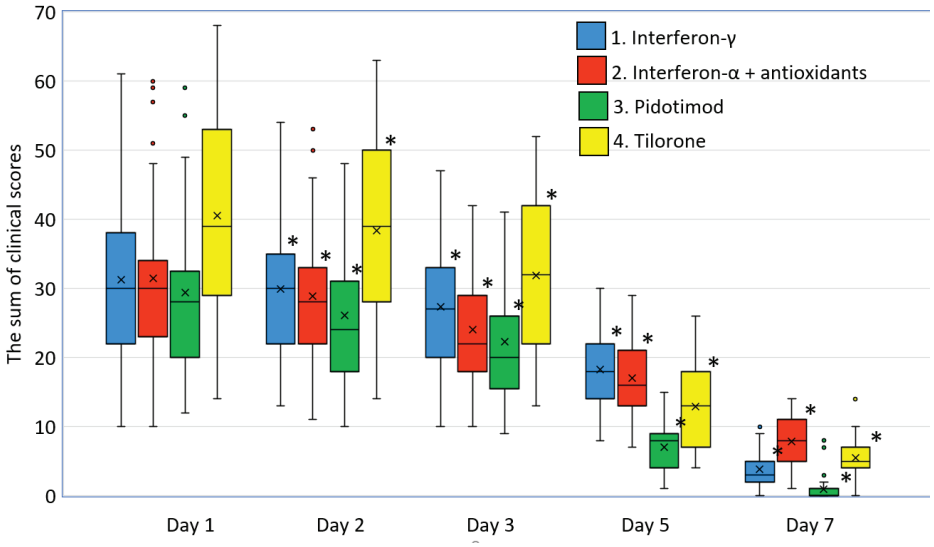

Figure. The dynamics of regression of ARI clinical manifestations in patients with concomitant allergic rhinitis who received different variants of antiviral therapy: × – mean values, points – outliers, *Me* (Q_1–Q_3; *Min–Max*)

* $p < 0.01$ compared with the parameters on the first day of the study (Wilcoxon test).

All the patients included in the study tolerated the treatment well. No cases of adverse events were noted. No patients were excluded or withdrawn from the study.

A statistically significant decrease in the total score for ARI clinical manifestations was observed in all the groups on day 2 of the treatment. At the same time, the severity of the disease in the cohort of patients treated with tilorone remained at a higher level compared with other groups (Figure, Table 2).

Subsequently, the main symptoms of the disease steadily regressed at approximately the same rate in patients with all the treatment options.

Table 2

Statistically significant intergroup differences		
Duration of the study, day	$p_{1,2,3,4}$ (the Kruskal – Wallis test)	The Dunn's test
1	0.008	$p_{1\text{-}4} = 0.039$, $p_{3\text{-}4} = 0.008$
2	0.001	$p_{1\text{-}4} = 0.047$, $p_{2\text{-}4} = 0.045$, $p_{3\text{-}4} = 0{,}001$
3	<0.001	$p_{2\text{-}4} = 0.032$, $p_{3\text{-}4} = 0.002$
5	<0.001	$p_{1\text{-}3} < 0.001$, $p_{1\text{-}4} = 0.002$, $p_{2\text{-}3} < 0{,}001$
7	<0.001	$p_{1\text{-}2} < 0.001$, $p_{1\text{-}3} < 0.001$, $p_{2\text{-}3} < 0.001$, $p_{3\text{-}4} < 0.001$

On day 5, the total score for patients in the tilorone group was lower than that in the IFN-γ cohort, and the pidotimod group had the highest regression rate of the main ARI symptoms. By day 7, the total score in the pidotimod group tended to zero and was significantly lower than in each of the other three groups, in which, despite a rapid decrease in the severity of symptoms, the residual ARI manifestations still persisted more clearly (Figure).

Since the pharmacological activity of all the studied drugs is largely associated with type I and type II IFN signals, we studied how the concentrations of IFN-α and IFN-γ in the blood serum changed as a result of different treatment options, the ability of blood cells to produce these cytokines *ex vivo*, and the proportion of peripheral blood lymphocytes expressing type I and type II IFN receptors.

A tendency toward an increase in the concentration of IFN-α in the blood serum of most ARI patients before the treatment was noted, and in the IFN-α + AO group, there was a significant increase in this parameter compared with healthy donors. The cohorts of patients were initially heterogeneous according to this criterion, as the level of IFN-α in the IFN-γ and pidotimod groups was lower than in the IFN-α + AO group. After the treatment, the concentration of IFN-α in the blood serum significantly decreased in patients of all groups and was in the range similar to the conditional norm (Table 3).

The content of IFN-α and IFN-γ in the supernatant of unstimulated peripheral blood cell cultures in the vast majority of cases (more than 75%) was below the detection limit in both healthy donors and patients with ARI. In addition, the sensitivity of the used test system was not enough to detect IFN-γ in the blood serum of more than 80% of patients and healthy people (data not provided).

NDV-induced production of IFN-α by blood cells *in vitro* in the patients from the IFN-γ, IFN-α + AO, and tilorone groups was initially higher than in healthy donors. In the pidotimod cohort, we only observed an upward trend for this parameter. No significant intergroup differences were detected before the treatment. After the treatment, the induced production of IFN-α in all the groups decreased; in the meantime, it was higher in the IFN-γ group than in the tilorone cohort and was at the level of a mathematically confirmed trend (Table 3).

Prior to the treatment, we revealed a decrease in the production of IFN-γ induced by PHA in the pidotimod group and a tendency toward a decrease in this parameter in other cohorts of patients. After the treatment, the induced production of IFN-γ increased to a level similar to the conditional norm, but this increase was statistically significant only in the IFN-α + AO and pidotimod groups. There were no mathematically confirmed intergroup differences both before and after the treatment (Table 3).

The proportion of lymphocytes expressing type I IFN receptor subunit 2 (CD118) in the peripheral blood in all groups of patients was initially higher than the conditional norm. After the treatment, a decrease in this parameter was observed in the tilorone group, which nevertheless remained at a higher level than in healthy donors. In other cohorts, the relative number of $CD118^+$ lymphocytes did not change.

When determining the proportion of lymphocytes expressing the IFN-γ receptor α-chain (CD119), no differences were found either between the groups of patients or between each of them and the conditional norm. Additionally, no significant changes were revealed in all the cohorts. At the same time, a slight upward trend in the proportion of $CD119^+$ and $CD118^+$ lymphocytes in the IFN-α + AO group was noted, that did not reach the level of the statistically confirmed trend (Table 3).

DISCUSSION

Higher baseline severity of ARI symptoms in the group of patients treated with tilorone makes it difficult to compare the clinical efficacy of this drug with that of other treatment options. However, a delay in the regression of symptoms in the first two to three days of the treatment was observed in the tilorone group, after which the clinical manifestations of ARI quickly faded away. This was probably due to the fact that tilorone at the first dose (on day 1) and second dose (on day 2) could act as an inducer of the production of not only IFNs of all types, but also other, mainly proinflammatory, cytokines. On the contrary, subsequent doses of this drug, taken on days 4 and 6 of the treatment could cause temporary hyporeactivity of IFN-producing cells and proinflammatory cytokines, which contributed to rapid relief of symptoms reflecting a local and systemic inflammatory response. In general, the features of the changes in the ARI clinical manifestations during the tilorone

Table 3

Changes in the parameters of production and sensing of types I and II IFNs in patients with ARI with concomitant allergic rhinitis who received different treatment options, *Me* (Q_1–Q_3; *Min–Max*)

Parameter	Healthy donors (conditional norm)	The duration of the study and the therapy option								Statistical significance of differences (multiple comparisons)#		Statistical significance of differences (paired comparisons)#
		Before the treatment				After the treatment						
		IFN-γ	IFN-α + AO	Pidotimod	Tilorone	IFN-γ	IFN-α + AO	Pidotimod	Tilorone			
		1	2	3	4	5	6	7	8	The Kruskal – Wallis test	The Dunn's test	The Wilcoxon test
Concentration of IFN-α in the blood serum, pg /ml	0 (0– 3.5; 0– 19.2)	0 (0–6.6; 0–37.8)	6.8 (2.2–9.9; 0–18.1)*	1.5 (0–5.6; 0–32.5)	0 (0–6.5; 0–37.9)	0 (0–1; 0-16)	0 (0–2; 0–6)	0 (0–1.8; 0–12.6)	0 (0–1; 0–8)	$p_{1,2,3,4} = 0.019$	$p_{1-2} = 0.044$; $p_{2-3} = 0.066$	$p_{1-5} = 0.002$; $p_{2-6} < 0.001$; $p_{3-7} = 0.016$; $p_{4-8} = 0.023$
IFN-α production *in vitro* induced by NDV, pg / ml	171 (75– 259; 20– 310)	252 (129–446; 0–670)*	213 (111–398; 8–600)*	183 (124–335; 4–647)	199 (108–328; 0–600)*	159 (104–252; 20–475)	151 (74–242; 20–271)	114 (46–182; 0–287)	143 (79–190; 29–407)	$p_{5,6,7,8} = 0.099$	$p_{5-8} = 0.081$	$p_{1-5} < 0.001$; $p_{2-6} = 0.006$; $p_{3-7} = 0.004$; $p_{4-8} = 0.012$
IFN-γ production *in vitro* induced by PHA, pg / ml	150 (56– 227; 6– 416)	72 (21–172; 0–745)	48 (12–123; 0–879)	29 (16–82; 0–879)*	79 (27–173; 0–1,369)	108 (22–188; 0–891)	174 (22–453; 0–1,364)	158 (19–444; 0–1,365)	129 (22–429; 0–1,385)	–	–	$p_{2-6} = 0.03$; $p_{3-7} = 0.003$
The proportion of $CD118^+$ lymphocytes in the blood, %	76 (68– 85; 60– 92)	90 (83–92; 63–97)*	91 (88–93; 78–94)*	94 (84–95; 74–98)*	92 (88–93; 77–94)*	89 (83–93; 68–97)*	92 (89–93; 81–97)*	89 (86–92; 76–96)*	89 (82–92; 66–95)*	$p_{1,2,3,4} = 0.024$	$p_{1-3} = 0.015$	$p_{4-8} = 0.036$
The proportion of $CD119^+$ lymphocytes in the blood, %	85 (80– 90; 61– 96)	85 (80–90; 6–96)	83 (79–89; 43–96)	88 (85–94; 60–96)	85 (80–87; 72–94)	85 (80–91; 75–97)	86 (84–90; 80–93)	87 (80–90; 58–93)	84 (81–89; 69–95)	–	–	–

Note: AO – antioxidants, NDV – Newcastle disease virus, PHA – phytohemagglutinin, # – the value of p is indicated only in cases when $p < 0.1$. * $p < 0.05$ compared with healthy donors (Mann – Whitney test).

treatment confirm the hypothesis about the mechanisms of clinical efficacy of tilorone, formulated earlier [30], and are consistent with the results of a recent study on the antiviral and cytokine-modulating activity of this drug in the model of influenza *in vivo* [25].

Conditional leadership of pidotimod in the relief of ARI symptoms, which manifested from day 5 of the study, can be partly associated with slightly lower initial severity of the disease in the group of patients who received this immunostimulant. Nevertheless, high effectiveness of pidotimod is of great interest because it is significantly further from classical antiviral agents than other comparison medicines used in this study in terms of the main mechanisms of the pharmacological action. Unlike pidotimod, tilorone, which has an immune-mediated antiviral effect, as well as IFN-γ and IFN-α2b with antioxidants, which are systematized as immunostimulants according to the anatomical, therapeutic, and chemical classification of drugs, are usually considered for etiotropic therapy of viral infections along with direct-acting antivirals. But it was pidotimod, whose effectiveness in the complex treatment of allergic rhinitis [31], asthma [22], as well as in the prevention of ARI [19] was previously proven, and which had a pronounced therapeutic effect in the acute phase of respiratory infection in patients with allergy in this study.

Probably, the use of a systemically acting immunostimulant that shifts the balance of the prevailing immune response in the T2→T1 direction was more important in terms of accelerating the relief of ARI symptoms for patients with concomitant allergic rhinitis than the use of drugs with a more pronounced antiviral effect. The synthetic dipeptide pidotimod like some immunomodulators of bacterial origin has immunoregulatory (anti-inflammatory or immune dampening) effects [32]. In this regard, it seems promising to continue studies of this drug not only as a stimulant of anti-infective defense, but also as a method of complex treatment of allergic rhinitis and other diseases accompanied by persistent inflammation in the respiratory tract.

Similar dynamics of regression of ARI clinical manifestations was observed in the groups of patients who received intranasally IFN-γ or a combination of topical and rectal dosage forms of IFN-α2b + AO. This result is interesting, since IFN-γ locally used a key mediator of the T1 response with proinflammatory activity[33] was comparable in clinical efficacy with a combination of systemic and topical dosage forms of IFN-α2b, a cytokine with a more pronounced antiviral effect and anti-inflammatory potential [34]. This is another indirect confirmation of a great importance of the T2→T1 immune response polarization for the regression of ARI clinical manifestations in patients with concomitant allergic rhinitis.

After IFN-α2 or another type I IFN binds two subunits of the corresponding receptor on the surface of the target cell and initiates biochemical cascades aimed at protecting against viruses, the ligand – receptor complex is internalized by endocytosis. This complex, when in the endosome, continues to exert biological (antiproliferative, immunomodulatory) effects for some time and only then undergoes lysosomal degradation [35]. However, the signals leading to the production of IFN-stimulated virostatic proteins are transduced through type I IFN receptor mainly when it is located on the cell surface. The ability of different type I IFNs to carry the receptor inside the cell correlates with the degree of affinity of the ligand – receptor interaction [36]. IFN-α2 is characterized by high affinity for type I IFN receptor [37], second only to IFN-β in this respect [38].

The interaction of IFN-γ with the receptor also finally leads to internalization and intracellular degradation of the ligand – receptor complex [39]. IFN-γ decreases the expression of its receptor in target cells by mechanisms independent of endocytosis [40]. Type I IFNs are also capable of suppressing the expression of IFN-γ receptors both as a result of blocking the transcription of the α-chain gene in this receptor [41] and secondarily due to stimulation of IFN-γ production [17], leading to the above-mentioned ligand-induced mechanisms for reducing sensitivity to IFN-γ.

In theory, these features of type I and type II IFN signal transduction could lead to a temporary decrease in the number of both types of IFN receptors on the plasmalemma of different cells (including circulating lymphocytes) in patients treated with systemically acting IFN-α2b. This could reduce the effectiveness of the natural antiviral mechanisms dependent on type I and type II IFN. The results of this study disavow this assumption. An upward trend in the proportion of lymphocytes expressing

type I IFN receptor subunit 2 and IFN-γ receptor α-chain was revealed on the last day of the 7-day treatment in the IFN-α + AO cohort and was not observed in other groups. This can be explained by the recirculation of internalized receptors [39] and / or the presence of previously unidentified positive feedback mechanisms leading to the restoration of the number of receptors after a ligand-induced decrease in their density on the surface of target cells. The results obtained are consistent with the data that it is IFN-α2b, but not IFN-β, that stimulates the recirculation of the internalized type I IFN receptor subunit 2 to the cell surface [42].

CONCLUSION

Generally similar clinical efficacy of IFN-γ in the nasal dosage form, the combination of rectal and nasal dosage forms of IFN-α + AO, pidotimod, and tilorone in the treatment of ARI in patients with allergic rhinitis was established. The results of this study allow to make a conclusion that drugs capable of polarizing the immune response in the T2→T1 direction are a useful option for the treatment of ARI in patients with concomitant allergic respiratory diseases. In this context, the choice of all the drugs investigated in this work should be recognized as justified. From the data obtained, a rational vector for the development of new effective agents for the pathogen-specific (etiotropic) treatment of ARVI in patients with respiratory allergy emerges: the search for natural and synthetic pharmacological substances that have both antiviral and T2→T1 properties.

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Evaluation of the cytotoxic activity and toxicity of a tropolone derivative with a potential antitumor effect

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ABSTRACT

The aim. To study the toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone *in vitro* and *in vivo*.

Materials and methods. 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was synthesized using a method for expanding the *o*-quinone cycle during the reaction between 5-nitro-2,6,8-trimethyl-4-chloroquinoline and 3,4,5,6-tetrachloro-1,2-benzoquinone while boiled in dioxane. An *in vitro* experiment was carried out in the human A549 cell line. Cell viability was assessed using the MTT colorimetric assay by reducing the optical density of the experimental samples compared with the control ones. Acute toxicity was studied on 20 BALB/c Nude male mice. The test compound was administered once orally as a suspension in 1% starch gel at three doses: 0.0055 (group 1), 0.055 (group 2) and 0.55 mg / g (group 3). The control group (group 4) received a placebo.

Results. We synthesized a new compound, 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone. Its structure was established by ^{1}H nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and mass spectrometry. The yield was 19.8 g (52%), the melting point was 205–207 ºC, bright yellow crystals (benzene) were observed. The half-maximal inhibitory concentration (IC_{50}) of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was 0.21 ± 0.01 μM, which was significantly lower ($p < 0.05$) than the IC_{50} of cisplatin (3.84 ± 0.23). Following the *in vivo* experiment, no toxic effect of tropolone was detected when administered once at a dose of 0.0055, 0.055, and 0.55 mg / g.

Conclusion. 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone demonstrated cytotoxic effects on the A549 cell line at a lower IC_{50} than cisplatin which is widely used in treatment of cancers, including lung cancer. Insolubility of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in water and the absence of its toxic effect in the studied modes determine the scope of its application for further study of cumulative and antitumor effects.

Keywords: tropolones, antitumor effect, human non-small-cell lung cancer A549 cell line, MTT assay

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

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Conformity with the principles of ethics. The study was approved by the local Bioethics Committee at the National Medical Research Center of Oncology (Protocol No. 1/61 of 19.02.2019).

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Оценка цитотоксической активности и токсичности производного тропалонов с потенциальным противоопухолевым действием

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РЕЗЮМЕ

Цель – исследование токсичности 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона *in vitro* и *in vivo*.

Материалы и методы. Для синтеза 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона использован метод расширения о-хинонового цикла в процессе реакции между 5-нитро-2,6,8-триметил-4-хлорхинолином и 3,4,5,6-тетрахлор-1,2-бензохиноном при кипячении в диоксане. Эксперимент *in vitro* проведен на клеточной линии рака легкого человека A549. Оценку жизнеспособности клеток проводили при помощи МТТ-колориметрического теста по уменьшению оптической плотности опытных проб по сравнению с контрольными. Исследование острой токсичности проведено на 20 самках мышей линии Balb/c Nude. Исследуемое соединение вводили однократно перорально в форме суспензии в 1%-м крахмальном геле в трех дозах: 0,0055 (1-я группа), 0,055 (2-я группа) и 0,55 мг/г (3-я группа). Контрольная группа (4-я) получала плацебо.

Результаты. Получен 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалон по ранее разработанному методу, его строение установлено данными ядерно-магнитного-резонанса ^{1}H и инфракрасной и масс-спектрометрии. Выход составил 19,8 г (52%), температура плавления 205–207 °С, ярко-желтые кристаллы (бензол). Ингибирующая концентрация IC_{50} 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона была равна 0,21 ± 0,01 мкМ, что оказалось статистически значимо меньше ($p < 0,05$) ингибирующей концентрации IC_{50} цисплатина равной 3,84 ± 0,23 мкМ. В результате исследования *in vivo* не выявлено токсического действия тропалона при однократном введении в дозах 0,0055; 0,055 и 0,55 мг/г.

Заключение. Показано, что 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалон проявляет цитотоксическую активность в отношении клеточной линии A549 в более низкой ингибирующей концентрации IC50, чем цисплатин, широко применяющийся в лечении злокачественных новообразований, в том числе рака легкого. Нерастворимость в воде 2-(6,8-диметил-5-нитро-4-хлорхинолин-2-ил)-5,6,7-трихлор-1,3-тропалона и отсутствие его токсического действия в исследованных нами режимах определяют границы его использования для дальнейшего изучения кумулятивных и противоопухолевых эффектов.

Ключевые слова: тропалоны, противоопухолевый эффект, культура клеток немелкоклеточного рака легкого человека A549, МТТ-тест

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

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INTRODUCTION

In the modern world, cancer is considered one of the most severe diseases with frequent fatal outcomes [1]. In addition, low efficiency and low selectivity of cytotoxic drugs used in clinical practice are combined with many side effects [1] and a small range of effective doses [3]. The use of effective treatment methods is one of the key factors for improving the prognosis of the course of cancer [4]. Therefore, development and study of new anticancer drugs that combine high cytotoxic activity with minimal side effects remain relevant.

Along with well-known chemotherapeutic agents, some scientists and physicians propose to use certain podophyllotoxin derivatives, diterpenes, and alkaloids as medicines [5]. A promising group of substances with a wide range of biological activities, including antitumor ones, is non-benzenoid aromatic compounds – tropolones [6, 7]. Their most studied representatives are β-thujaplicin (hinoktiol), colchicine, and colchamine. There are some approaches to the synthesis of tropolones allowing to obtain a wide range of substances with various biological properties, such as antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and antitumor ones [5, 8].

2-[7-acetyl-9,11-di(tert-butyl)-4-methyl-5-chlorobenzo[b][1,4]oxazepino[7,6,5-de]-quinoline-2-yl]-5,6,7-trichloro-1,3-tropolone has the most similar structure to the studied compound; it shows cytotoxic activity against breast tumor cells MCF-7 and MCF-10 cell line, lung cancer cells Lu, liver cancer cells Hep-G2, and tumor epithelial cells KB [6]. Derivatives of 2-quinoline-2-yl-1,3-tropolones have shown activity against various cancer cell lines in the lungs (A549 and H441), ovaries (OVCAR-3 and OVCAR-8), colon (HCT 116), and pancreas (Panc-1) in the range from $IC_{50\ to\ 5}$ μM [9].

The aim of this study was to analyze the toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone *in vitro* and *in vivo*.

MATERIALS AND METHODS

Studied compound. The structural formula of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone is presented in

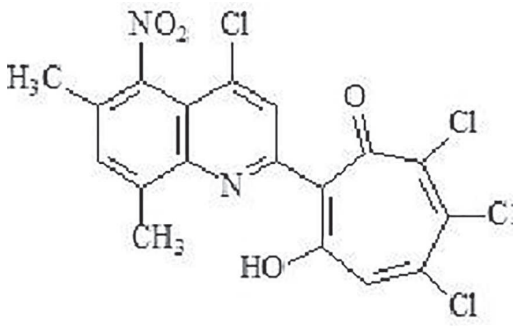

Fig. 1. Structural formula of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone

Fig. 1. Compound 1 (2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone) was synthesized using a method for expanding the *o*-quinone cycle [10]. The reaction proceeded between 5-nitro-2,6,8-trimethyl-4-chloroquinoline (2) and 3,4,5,6-tetrachloro-1,2-benzoquinone (**3**) while boiled in dioxane (Fig. 2)

Cytotoxic activity testing. The experiment was carried out in the human non-small-cell lung cancer A549 cell line. Cell viability was assessed using the MTT colorimetric assay. The cells were cultured in a 96-well plate under standard sterile conditions: temperature of +37 ºC, 5% CO2, DMEM culture medium, 10% FBS. The tested substance, the reference drug (cisplatin), and the solvent (DMSO) were added at the concentration of 0.004–2.226 μM. The cells continued to be incubated under the same conditions for 72 hours, after which 20 μL of the MTT solution

was added to them. Then the incubation was continued for another 2 hours. Formazan crystals formed as a result of the MTT assay were dissolved in DMSO, and the optical density (the average wavelength was 492 nm) was measured using the Stat Fax 2100 Microplate Reader (Awareness Technology, USA) [11].

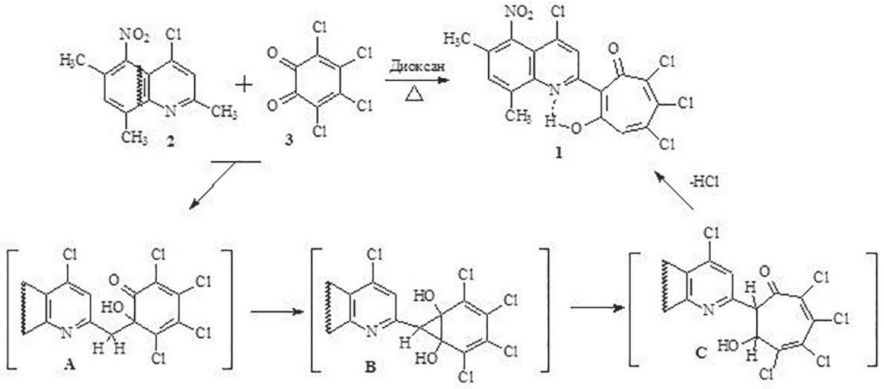

Fig. 2. Synthesis of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone

Acute toxicity testing. Acute toxicity was studied on 20 BALB/c Nude female mice weighing 25.5–27.5 g. They were divided into 4 groups of 5 animals each. The study was conducted on females because they are considered more sensitive to the effects of substances than males (GOST 32296-2013). Since the experiment included linear animals and followed the principles of the 3Rs, 5 animals were used in each group, which seemed possible for statistical analysis of the data [12].

The acute toxicity of tropolone was studied on BALB/c Nude mice, since this compound with a potential antitumor effect can be studied on xenograft models created on immunodeficient mice, which are considered more sensitive to the exposure [13, 14]. The studied compound was administered once orally as a suspension in 1% starch gel at three doses: 0.0055 mg / g (group 1), 0.055 mg / g (group 2), and 0.55 mg / g (group 3). The choice of a maximum dose was limited by the insolubility of the test compound in water. The control group (group 4) received 1% starch gel. After the administration of the substance, the animals were examined daily for 14 days. The body weight of the animals was determined on days 7 and 14. The following parameters were used to assess the dose-dependent effects of tropolone: survival rate, health disorders during daily observation, weight dynamics; at necropsy: deviations from the normal condition of the skin and visible mucous membranes, as well as deviations from normal size, shape, color, structure, and location of internal organs, body cavity effusions, and secretions from natural body orifices (GOST R 56701-2015). Animals were euthanized on day 14 of the experiment by cervical dislocation.

Statistical analysis of the data. To determine the inhibitory concentration when testing the cytotoxic activity of the compound, the proportion (%) of viable cells was calculated in the test wells relative to the positive control wells, which cell viability was considered as 100%. Logarithm of the concentration at 50% cell viability was calculated by the probit analysis, and then the half-maximal inhibitory concentration (IC50) was calculated [15]. In the statistical analysis of the results, the mean and the standard deviation $M \pm m$ were evaluated; the Wilcoxon – Mann – Whitney test and the Kruskal – Wallis test with the post-hoc Dunn's test were used. The GraphPad Prism 5.0 software was used for statistical analysis of the results.

RESULTS

2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone was obtained according to the previously developed method [16], and its structure was established by ^{1}H nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and mass spectrometry. When assessing the cytotoxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone against the A549 cell culture, its IC_{50} was determined. It reached 0.21 ± 0.01 μM, which turned out to be statistically significantly lower than the IC_{50} of cisplatin equal to 3.84 ± 0.23 μM (the differences were statistically significant compared with the control group ($p < 0.05$, according to the Kruskal – Wallis test), widely used in clinical practice [17].

The analysis of the acute toxicity of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-tri-

chloro-1,3-tropolone with its single administration at the dose of 0.0055 mg / g (group 1), 0.055 mg / g (group 2), and 0.55 mg / g (group 3) showed 100% survival rate, which did not allow to detect a lethal dose. Administration of higher doses of the studied substance was impossible because the volume fraction of the solid dispersed phase exceeded that of the liquid dispersed phase, and the substance was water-insoluble. No health disorders were registered during 14 days of daily observation in all four groups of animals. Necropsy showed no pathological changes in most mice. In group 2, two mice had single focal hemorrhages up to 1 mm in diameter in the medial lobe of the liver. In group 3, two animals had hyperemia in the liver, and one animal had focal pulmonary hemorrhage. Pathological changes found at necropsy in some experimental mice could result from the toxic effect of tropolone or from euthanasia.

The analysis of the weight dynamics in mice with a single tropolone administration showed its slight decline by the end of the observation period in only one animal from group 3. In the other animals, on the contrary, an increase in weight by 0.5–2 g was observed by the end of the experiment compared with the baseline values. Such an increase was most pronounced in the control group and in group 1, where the mice received a minimum compound dose of 0.0055 mg / g. The weight dynamics in the animals of these groups was significantly different from that in group 3 with mice receiving the substance at the maximum studied dose of 0.55 mg / g (Table).

Table

Weight dynamics in BALB/c Nude mice on day 14 of the experiment after a single dose of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone, $M \pm m$

Group	Body weight, g
1, $n = 5$	1.6 ± 0.11▫
2, $n = 5$	1.3 ± 0.29
3, $n = 5$	0.8 ± 0.38*•
4, controls, $n = 5$	1.7 ± 0.14▫

Note: * differs from values in the control group, $p < 0.01$; • differs from values in group 1, $p < 0.01$, ▫ differs from values in group 3, $p < 0.01$; Wilcoxon – Mann – Whitney test.

The above intergroup differences may be associated with metabolic and other changes in the body of mice under the influence of the studied substance.

DISCUSSION

A number of studies demonstrated high antitumor effect of tropolones together with their minimal effect on normal tissues and low toxicity, which makes this group of compounds promising [18]. To date, hinoktiol (β-thujaplicin) is the most studied tropolone, which exhibits high antitumor activity against various cancer cell lines [19, 20].

L. -H. Li et al. determined the viability of A549 tumor cells exposed to hinoktiol as 52.7 ± 3.6 and 34.7 ± 5.2 when exposed for 48 hours at a concentration of 5 and 10 μM, respectively, and 28.9 ± 1.1 and 18.2 ± 7.2 when exposed for 72 hours at a concentration of 5 and 10 μM, respectively [21]. H. Wakabayashi et al. demonstrated that IC50 of synthesized compounds from the group of tropolones, 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone, against human oral squamous cell carcinoma cell lines HSC-2, HSC-3, and HSC-4 varied from 31 to 450 μM depending on the incubation time (24, 48, 72, and 96 hours), which was significantly higher than the IC50 of the tropolone we synthesized [22]. Our results are consistent with the data of other researchers and may indicate high antitumor efficacy of a new compound, 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone.

A number of studies explained high antitumor efficacy of hinoktiol. L. -H. Li et al. confirmed that hinoktiol induces autophagy, cell cycle arrest in the S-phase, and cellular aging in lung cancer cells and inhibits cell proliferation. Thus, hinoktiol, probably like other substances from the tropolone group, can act as an effective anticancer agent due to induction of DNA damage, autophagy, cell cycle arrest, and cellular aging [21].

A comparison of the acute toxicity of our synthesized compound with that of other analogous substances showed its minimal toxic effect on the body of laboratory animals. Thus, LD50 for γ-thujaplicin, β-dolabrin, and hinoktiol were 277 mg / kg, 232 mg / kg, and 191 mg / kg, respectively [23]. Y. Morita et al. determined LD50 for 4-acetyltropolone, hinoktiol, β-dolabrin, γ-thujaplicin, and α-thujaplicin, which are 335.2; 191; 232; 277, and 256 mg / kg, respectively [24]. In our study, the acute toxicity of 7-bromo-2(4-hydroxyanilino)-tropone and 4-isopropyl-2-(2-hydroxyanilino)-tropone was not detected.

CONCLUSION

The MTT assay showed that 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone exhibited cytotoxic activity against the human non-small-cell lung cancer A549 cell line, and

the IC50 of the proposed compound was lower than that of cisplatin.

Insolubility of 2-(6,8-dimethyl-5-nitro-4-chloroquinoline-2-yl)-5,6,7-trichloro-1,3-tropolone in water limited the range of its investigated doses and did not allow for determination of its lethal dose and toxicity class while studying the acute toxicity. The absence of pronounced toxic effect of tropolone administered at a single dose of 0.0055, 0.055, and 0.55 mg / g determines the scope of its use for further study of cumulative and antitumor effects.

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Kit O.I. – study design, interpretation and analysis of the results. Minkin V.I. – conception and design, synthesis of the substance. Lukbanova E.A. – interpretation and analysis of the results, drafting of the manuscript. Sayapin Yu.A. – synthesis of the substance, drafting of the manuscript. Gusakov E.A. – carrying out of 1H NMR and IR spectroscopies, mass spectrometry, analysis of the results. Sitkovskaya A.O. – carrying out of MTT assay, editing of the manuscript. Filippova S.Yu. – working with the cell culture, carrying out of MTT assay. Komarova E.F. – conception and design, editing of the manuscript. Volkova A.V. – carrying out of a probit analysis. Khodakova D.V. – statistical analysis. Mindar M.V. – drafting of the manuscript. Lazutin Yu.N. – editing of the manuscript. Engibaryan M.A. – technical editing of the manuscript. Kolesnikov V.E. – drafting of the manuscript, compilation of the references.

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An artificial intelligence computer system for differential diagnosis of lysosomal storage diseases

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ABSTRACT

Aim. To improve the efficiency of diagnosis of hereditary lysosomal storage diseases using an intelligent computer-based decision support system.

Materials and methods. Descriptions of 35 clinical cases from the literature and depersonalized data of 52 patients from electronic health records were used as material for clinical testing of the computer diagnostic system. Knowledge engineering techniques have been used to extract, structure, and formalize knowledge from texts and experts. Literary sources included online databases and publications (in Russian and English). On this basis, for each clinical form of lysosomal diseases, textological cards were created, the information in which was corrected by experts. Then matrices were formed, including certainty factors (coefficients) for the manifestation, severity, and relevance of signs for each age group (up to 1 year, from 1 to 3 years inclusive, from 4 to 6 years inclusive, 7 years and older). The knowledge base of the expert system was implemented on the ontology network and included a disease model with reference variants of clinical forms. Decision making was carried out using production rules.

Results. The expert computer system was developed to support clinical decision-making at the pre-laboratory stage of differential diagnosis of lysosomal storage diseases. The result of its operation was a ranked list of hypotheses, reflecting the degree of their compliance with reference descriptions of clinical disease forms in the knowledge base. Clinical testing was carried out on cases from literary sources and patient data from electronic health records. The criterion for assessing the effectiveness of disease recognition was inclusion of the verified diagnosis in the list of five hypotheses generated by the system. Based on the testing results, the accuracy was 87.4%.

Conclusion. The expert system for the diagnosis of hereditary diseases has shown fairly high efficiency at the stage of compiling a differential diagnosis list at the pre-laboratory stage, which allows us to speak about the possibility of its use in clinical practice.

Keywords: hereditary diseases, orphan diseases, lysosomal storage diseases, differential diagnosis, expert system, decision support, certainty factors

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Компьютерная система для дифференциальной диагностики лизосомных болезней накопления на основе методов искусственного интеллекта

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РЕЗЮМЕ

Цель – повышение эффективности диагностики наследственных лизосомных болезней накопления с использованием интеллектуальной компьютерной системы поддержки принятий решений.

Материалы и методы. В качестве материала для клинической апробации компьютерной диагностической системы использованы описания 35 клинических случаев из литературы и данные 52 пациентов из электронных медицинских карт (в деперсонифицированном виде). Методы инженерии знаний использовались для извлечения, структуризации и формализации знаний из текстов и у экспертов. Литературные источники включали онлайн-базы данных и публикации (русско- и англоязычные). На этой основе для каждой клинической формы лизосомных болезней были сформированы текстологические карты, информация которых корректировалась экспертами. Затем формировались матрицы, включающие факторы уверенности (коэффициенты) для манифестации, выраженности и релевантности признаков по каждой из возрастных групп (до 1 года, от 1 года до 3 лет включительно, от 4 до 6 лет включительно, 7 лет и старше). База знаний экспертной системы реализована на онтологической сети и включает модель заболевания с эталонными вариантами клинических форм. Принятие решений осуществляется с использованием продукционных правил.

Результаты. Разработана экспертная компьютерная система поддержки принятия клинических решений на долабораторном этапе дифференциальной диагностики лизосомных болезней накопления. Результатом ее работы является ранжированный перечень диагностических гипотез, отражающий степень их соответствия эталонным описаниям клинических форм болезней в базе знаний. Проведена апробация системы на случаях из литературных источников и на данных пациентов из электронных медицинских карт. Критерием для оценки эффективности распознавания болезни было вхождение верифицированного диагноза в перечень из пяти гипотез, выдаваемых системой. По итогам проведенной апробации точность составила 87,4%.

Заключение. Экспертная система для диагностики наследственных болезней показала достаточно высо-

кую эффективность на этапе формирования дифференциально-диагностического ряда на долабораторном этапе, что позволяет говорить о возможности ее использования в клинической практике.

Ключевые слова: наследственные заболевания, орфанные болезни, лизосомные болезни накопления, дифференциальная диагностика, экспертная система, поддержка принятия решений, факторы уверенности

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

Источник финансирования. Исследование проведено в рамках финансирования государственного задания «Системы искусственного интеллекта, извлечение знаний и анализ текстов 2019–2023» (№ 0063-2019-0001).

Соответствие принципам этики. Для публикации результатов оригинальной работы использовались деперсонифицированные ретроспективные данные из электронных медицинских карт.

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INTRODUCTION

Lysosomal storage diseases (LSDs) [1,2], including mucopolysaccharidoses (MPS), mucolipidoses (ML), gangliosidoses (GS), and other forms, belong to the class of hereditary diseases and are characterized by accumulation of a specific substrate. The disease begins at birth and has progressive nature, which causes an increase in the severity of phenotypic characteristics with age [3]. The importance of the earliest possible diagnosis of these diseases has especially increased recently due to the advent of enzyme replacement therapy [4–7]. With pharmacological replacement of the deficient enzyme, the progression of pathological manifestations stops, however, reduction of the changes that occurred earlier does not occur.

At the same time, the diagnosis of LSDs in children in early stages of disease manifestations can be extremely difficult due to high variability in the clinical presentation. Therefore, conflicting descriptions are found in various literary sources, and personal experience of the physician in providing care for these patients is very limited. However, early suspicion of a rare disease in children requires knowledge about minor disease manifestations. Nonspecific initial symptoms lead to long-term failure to diagnose the disease or to its misdiagnosis [8]. For example, in the Netherlands, the timing of diagnosis for patients with MPS did not change between 1988 and 2017, and there is still a long delay between the first visit to the physician with complaints about disease symptoms and the final diagnosis [9]. However, a great number of diseases similar to LSDs in phenotypic manifestations makes it difficult for the physician to compare patient's symptoms with descriptions in clinical guidelines, monographs, articles, and databases.

At the same time, it is possible to identify certain patterns of signs that create a certain "portrait" of the disease. To accelerate and improve the accuracy of the identification of orphan diseases, it is possible to use artificial intelligence computer-based decision support systems. Among the effectively used in the past and currently existing software tools, the Russian DIAGEN [10], the French GENDIAG [11], the Australian POSSUM [12], the British Face2Gene [13], and the German Ada DX [14] are worth noting. All of them use expert knowledge to some extent, although the principles of its construction differ.

It was shown that such systems can increase the likelihood of early recognition of orphan diseases. At the same time, there are several reasons that seriously hinder the use of foreign-produced software products:

– partial inconsistency with the accepted Russian terminology,

– features of ethnic diversity, which are superimposed on the phenotypic manifestations of diseases,

– requirements to protection of patients' personal data, since some of the foreign-made systems are implemented as cloud applications on foreign servers.

In addition, an important aspect is interpretation of the diagnostic solutions offered by the system.

The absence or formal explanation does not contribute to the understanding of the proposed diagnostic hypotheses. This makes the development of a Russian-made computer-based system for the diagnosis of hereditary diseases relevant.

The aim of this study was to improve the efficiency of diagnosis of genetic disorders using an intelligent clinical decision support system that compiles a limited differential diagnosis list at the pre-laboratory stage of patient examination.

MATERIALS AND METHODS

When elaborating a computer system for the differential diagnosis of LSDs, the main task was to create a knowledge base. To do this, first, an analysis of literary sources was carried out: monographs and publications in Russian and English, with a particular emphasis on descriptions of cases from clinical practice, Russian clinical guidelines, and online databases on the area of interest. They served as the primary material for the creation of a knowledge-based system. The knowledge obtained from literary sources was structured using a specially developed form – a textological card [15], which recorded not only the fact of symptom detection, but also the period of its manifestation, its severity, and the frequency of its occurrence for a particular diagnosis indicated by the authors. These structured descriptions of diseases, aggregating knowledge from a variety of sources, were subsequently used by the experts in the formation of symptom complexes describing the differentiated LSDs.

The experts identified relevant phenotypic signs and indicated certainty factors characterizing their level of confidence in the manifestation of symptoms at a certain age. Some manifestations were represented by more general concepts, such as cardiopathy, due to the occurrence of various signs characterizing morphological or functional changes. Four age groups were identified in which manifestation and / or changes in modality (diagnostic significance or relevance) and severity of signs in LSDs were noted: the first year of life, from 1 to 3 years inclusive, from 4 to 6 years inclusive, 7 years and older. Each sign was accompanied by three expert assessments: modality coefficients and certainty factors for manifestation and degree of expression.

Thus, knowledge engineering methods were used to extract, structure, and formalize knowledge, on the basis of which the knowledge base of the expert system was created [16].

Descriptions of the clinical presentation of diseases in 87 patients with verified diagnoses were the material for the clinical testing of the system. The sample included 35 clinical cases from the literature (MPS – 27, GS – 5, ML – 3) and depersonalized formalized data from health records of 52 patients (MPS – 46, ML – 6) from the Department of Congenital and Hereditary Diseases of the Veltishchev Research Clinical Institute for Pediatrics of the Pirogov Russian National Research Medical University and the Medical Genetic Center of the Moscow Regional Research and Clinical Institute.

RESULTS

An intelligent (expert) GenDiES system was developed to support clinical decision making at the pre-laboratory stage of diagnosing hereditary LSDs. Knowledge base rules are implemented using the ontological approach. In a problem solver, production rules may contain signs that are not classified by experts as diagnostically significant for the hypothesis under consideration. The presence of such signs in the model does not reject the diagnosis but leads to a decrease in the rank of the hypothesis in the differential list. An integrated assessment model [17] allows to take into account expert assessments of modality, manifestation, and severity of signs and compares a new object with reference variants of the known clinical forms. Based on the detected signs, the model provides calculations to compare new cases of LSDs with reference descriptions of these diseases. As a result, a differential diagnosis list is compiled.

The GenDiES system problem solver includes several steps required to generate and validate hypotheses. At the first step, selection of diagnoses takes place which have no “against” signs in the patient’s description or signs noted by experts as contradicting a group or a subgroup of diseases. An example of this group of signs is the “cherry-red spot of the macula”, which immediately allows to exclude the MPS group. At the second step, the remaining potentially possible diagnoses for the patient are ordered by the number of signs “not related” to the hypothesis – in ascending order – from zero and then with an interval of one. A sign “not related” to the hypothesis is a sign that is not included in a list of signs for a particular clinical form as a diagnostically significant one, but was not listed as an exclusion sign. At the third

step, a series of integrated assessments for expert certainty factors for signs in a certain clinical case is formed according to the proposed diagnostic hypotheses. Then, personal integrated assessments are compared with the reference ones for clinical forms of LSDs and the percentage of coincidence is calculated. Hypotheses are ranked by the percentage of coincidence with the reference descriptions, starting with the most similar one. This ranked list of the first five hypotheses is fed to the output of the system. However, at the request of the physician, this list can be expanded.

As an explanation for each hypothesis put forward, the physician receives information about the patient's signs, grouped into the following categories, depending on their importance: main, necessary, secondary. Separately, the user is provided with information about the signs observed in the patient, but not included by the experts in the symptom complex of this disease in the GenDiES system. The physician also receives a list of signs characteristic of this clinical form, but not detected in the patient. This allows to direct the attention of the physician to the search for additional signs in the patient, the presence of which could increase the level of confidence in this diagnosis.

According to the results of the expert system testing on 87 cases of MPS, ML, and GS, the accuracy of including diagnoses in a limited differential diagnosis list was 87.4%; i.e. in 76 cases, the correct diagnosis (corresponding to the verified one) was among the first five hypotheses at the pre-laboratory stage of diagnosis.

It is equally important to analyze 11 erroneous diagnostic hypotheses using the GenDiES system, which were distributed by clinical forms as follows: MPS III – 3, MPS IV – 5, MPS VI – 1, MPS VII – 1, ML III – 1. Of 9 patients diagnosed with MPS III (Sanfilippo syndrome), in 3 cases (age: 4 years, 7 years 3 months, 7 years 8 months), this clinical form was not listed among the first five possible hypotheses due to the absence of signs of scaphocephaly, pectus carinatum, kyphoscoliosis, and hand joint deformities in the reference descriptions. In 5 cases of MPS IV (Morquio syndrome), the correct diagnostic hypothesis was not included in the first five due to the presence of splenomegaly in the clinical presentation in all patients, which was also absent in the reference description in the system. Patients diagnosed with MPS IV were aged 2 years 3 months, 6 years 11 months, 8 years, 8 years 9 months, and 9 years 2 months. A patient with MPS VI at the age of 1 year 1 month was characterized by an early manifestation of coarse facial features and lumbar hyperlordosis, as well as by the presence of an uncharacteristic sign – pectus excavatum. A patient with MPS VII at the age of 6 months already had signs that usually appear much later: hypertrichosis, corneal opacity, hepatomegaly, splenomegaly, and cardiopathy.

The described phenotypic signs according to the literature, including clinical guidelines, are extremely rare or absent. In a patient aged 5 years and 8 months diagnosed with ML III, the correct hypothesis was not included in the limited list of diagnoses, while the first place was taken by the hypothesis that the patient had phenotypically very similar ML II.

At the same time, it should be noted that in all 11 cases, the diagnoses corresponding to the verified ones were presented in the list of the ranked hypotheses, but below the fifth place. They were presented in the differential diagnosis lists containing 10 possible diagnoses.

DISCUSSION

Hereditary LSDs are characterized by similar phenotypic manifestations, but differences in the timing of manifestations, degree of intensity, and diagnostic significance of signs can help identify these diseases at the pre-laboratory stage of diagnosis. However, the rarity of this pathology in the practice of a pediatrician does not allow him to remember the signs and various combinations of manifestations for individual clinical forms, depending on the age of the patient [18].

Help in improving the accuracy and timeliness of diagnosis can be provided by computer-based clinical decision support systems. At the stage of pre-laboratory diagnosis, they make it possible to form a differential diagnosis list. In different systems, this field of hypotheses is different. In the previously used Russian DIAGEN [10] and French GENDIAG [11] systems, the physician was offered an ordered limited list of three to five diagnostic hypotheses. In the new German system Ada DX [14], the correct diagnosis is found among the five most appropriate variants of the disease in 53.8% of cases, and as the most appropriate variant of the disease – in 37.6% of cases. In contrast, the British system Face2Gen [13] derives all possible hypotheses, supplementing them with probabilistic estimates.

Based on domestic and foreign best practices, when creating the GenDiES system, it was decided to form a list of five hypotheses proposed to the physician with a possibility of expanding it to ten or more. However, the expansion of the differential diagnosis list will lead to inclusion of less probable diagnostic hypotheses in it.

CONCLUSION

The developed expert system GenDiES for support of clinical decision making at the pre-laboratory stage of diagnosing LSDs demonstrated efficiency of 87.4% in the formation of a limited differential diagnosis list of five hypotheses. The proposed approach to the extraction of knowledge, accompanied by expert assessments, and the implemented mathematical model of the artificial intelligence system have shown their effectiveness and possibility of application in clinical practice. The system is open and allows to expand the knowledge base for the diagnosis of other hereditary diseases.

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Authors contribution

Kobrinskii B.A. – conception and design, analysis and interpretation of the results, final approval of the manuscript for publication, substantiation of the manuscript or critical revision of the manuscript for important intellectual content. Blagosklonov N.A. – design of the study, analysis and interpretation of the results, drafting of the manuscript. Demikova N.S. – analysis and interpretation of the results, substantiation of the manuscript or critical revision of the manuscript for important intellectual content. Nikolaeva E.A., Kotalevskaya Y. Y. – collection of data and drafting of the manuscript. Melikyan L.P., Zinovieva Y. M. – collection of data.

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Relationship of the expression of calcium-handling proteins in the sarcoplasmic reticulum with polymorphic variants of their genes and with structural and functional parameters of the heart in patients with atrial fibrillation

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ABSTRACT

Aim. To investigate the relationship between the expression of Ca^{2+} handling proteins of the sarcoplasmic reticulum, polymorphic variants of their genes, and structural and functional parameters of the heart in patients with atrial fibrillation (AF).

Materials and methods. The study included patients with AF. The patients underwent radiofrequency ablation, during which a myocardial biopsy was taken. The patients underwent echocardiography (EchoCG) before surgery. Polymorphic variants rs1860561 of the *ATP2A2* gene and rs6684209 and rs7521023 of the *CASQ2* gene were determined in the patients by real-time polymerase chain reaction (PCR), and the level of expression of SERCA2a and CASQ2 proteins in the myocardium was detected by immunoblotting.

Results. Carriers of the GG genotype at rs1860561 of the *ATP2A2* gene and CC genotype at rs6684209 of the *CASQ2* gene were characterized by significantly higher expression of the corresponding proteins. Using cluster analysis, we identified groups of patients by the level of SERCA2a and CASQ2 expression: group 1 – patients with low protein content; group 2 – patients with high protein content. According to clinical and anamnestic parameters, the patients in the selected groups were homogeneous. In patients with high SERCA2a levels, the end systolic and diastolic volumes of the left ventricle (LV) were significantly higher than those in patients with low levels of this protein. The rates of early (peak E) and late left ventricular diastolic filling (peak A) were significantly lower in the group with high SERCA2a expression. A comparative analysis of EchoCG data of patients distributed by the level of CASQ2 expression in the myocardium did not reveal significant differences between the groups.

Conclusion. The polymorphic variant rs1860561 of the *ATP2A2* gene and rs6684209 of the *CASQ2* gene can modulate the level of SERCA2a and CASQ2 expression. SERCA2a expression is associated with the functional and structural parameters of the heart in patients with AF.

Keywords: atrial fibrillation, Ca^{2+}-ATPase of the sarcoplasmic reticulum, calsequestrin, polymorphic variants of genes, echocardiography

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

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at the Cardiology Research Institute, Tomsk NRMC (Protocol No. 139 of 18.11.2015).

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Сопряженность экспрессии кальций-транспортирующих белков саркоплазматического ретикулума с их полиморфными вариантами генов и структурно-функциональным состоянием сердца пациентов с фибрилляцией предсердий

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РЕЗЮМЕ

Цель. Исследовать взаимосвязь между экспрессией Ca2+-транспортирующих белков саркоплазматического ретикулума, полиморфными вариантами их генов и структурно-функциональным состоянием сердца пациентов с фибрилляцией предсердий (ФП).

Материалы и методы. В исследование включили пациентов с ФП. Больным проведена радиочастотная аблация, во время которой была взята биопсия миокарда. Пациентам проводили эхокардиографию (ЭхоКГ) до оперативного вмешательства. У больных определены полиморфные варианты rs1860561 гена *ATP2A2* и rs6684209, rs7521023 гена *CASQ2* методом полимеразной цепной реакции в режиме реального времени и уровень экспрессии белков SERCA2a и CASQ2 в миокарде методом иммуноблоттинга.

Результаты. Для носителей генотипов GG rs1860561 гена *ATP2A2* и CC rs6684209 гена *CASQ2* характерны значимо более высокие экспрессии соответствующих белков. С помощью кластерного анализа были выявлены группы пациентов по уровню экспрессии SERCA2a и CASQ2: 1 – пациенты с низким содержанием белков; 2 – с высоким содержанием белков. По клинико-анамнестическим показателям пациенты отобранных групп оказались практически однородны. У пациентов с высоким уровнем SERCA2a величины конечного систолического и диастолического объемов левого желудочка (ЛЖ) были значимо больше, чем таковые у больных с низким уровнем этого белка. Скорости раннего (пик Е) и позднего диастолического наполнения (пик А) ЛЖ были статистически значимо ниже в группе с высоким уровнем экспрессии SERCA2a. Сравнительный анализ данных ЭхоКГ пациентов, распределенных по уровню экспрессии CASQ2 в миокарде, не выявил значимых различий между группами.

Заключение. Генотипы rs1860561 гена *ATP2A2* и rs6684209 гена CASQ2 могут модулировать уровень экспрессии SERCA2a и CASQ2. Экспрессия SERCA2a сопряжена с функционально-структурными показателями сердца пациентов с ФП.

Ключевые слова: фибрилляция предсердий, Ca^{2+}-АТФаза саркоплазматического ретикулума, кальсеквестрин, полиморфные варианты генов, эхокардиография

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. Исследование одобрено локальным этическим комитетом НИИ кардиологии Томского НИМЦ (протокол № 139 от 18.11.2015).

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INTRODUCTION

In recent decades, the growth in the atrial fibrillation (AF) prevalence among the population of developed countries of the world has increased by 2–3 times [1]. According to the Framingham Heart Study, AF patients have a 1.5–2 times increased risk of annual mortality compared with the general population [2]. Despite significant advances in the study of AF mechanisms, the existing AF treatment standards have limited effectiveness [3]. It has been established that the molecular mechanisms of the triggered activity of cardiomyocytes are caused by impaired intracellular homeostasis of calcium ions [4]. One of the key functional proteins providing the transport of calcium ions in the cell is Ca^{2+}ATPase (SERCA2a) of the sarcoplasmic reticulum (SR). This protein is responsible for reuptake of calcium ions from the myoplasm into the SR [5]. A protein called calsequestrin (CASQ2) is responsible for binding of calcium ions inside the SR. In addition, CASQ2 affects the stability of the ryanodine receptor structure in the SR [6]. This suggests that CASQ2 is involved in the development of diastolic calcium leak.

The significance of the functional state of SERCA2a and CASQ2 in the mechanisms of AF initiation and maintenance has been shown both in experimental studies [7, 8] and in clinical trials [9, 10]. At the same time, the presence of AF is associated with both low and high levels of SERCA2a in the myocardium [9, 10]. Such a difference in the results may be due to the peculiarities of the examined samples of patients and, in particular, due to the different genetic variants of these proteins. Indeed, it was found that the genes encoding SERCA2a and CASQ2 have stable polymorphic variants which can affect the functional characteristics of these proteins. It was discovered that the presence of the polymorphic variant rs1860561 of the Ca^{2+}ATPase gene (*ATP2A2*) may be associated with a lower risk of life-threatening arrhythmias [11]. The involvement of CASQ2 in provoking sudden cardiac arrest due to ventricular arrhythmias may be associated with the polymorphic variant rs7521023 of the *CASQ2* gene [12]. However, the available data are insufficient to say whether these proteins and their genes are associated with the structural and functional parameters of the heart in AF patients.

The aim of the study was to assess the relationship of the expression of Ca^{2+}ATPase and calsequestrin, as well as the presence of polymorphic variants rs1860561 of the *ATP2A2* gene and rs7521023 and rs6684209 of the *CASQ2* gene with the structural and functional parameters of the heart in patients with AF.

MATERIALS AND METHODS

An observational, cross-sectional, uncontrolled study was carried out. The study included 45 patients with AF. The study was carried out in accordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee at the Cardiology Research Institute of Tomsk NMRC. All patients signed an informed consent to participate in the study. The average age of the patients was 43 [39; 48] years. The duration of AF was 3 [2.0– 4.0] years. As antiarrhythmic therapy, the patients received: amiodarone – 11 (24%), sotalol – 6 (13%), propafenone – 11 (24%), beta blockers – 7 (16%), and allapinin – 2 (4%).

At the time of hospitalization, 26 (58%) patients were receiving anticoagulant therapy, and 7 (16%) patients were receiving antiplatelet therapy. The incidence of hypertension was 31% (n = 14), and the incidence of coronary artery disease was 9% (n = 4). Upon admission, the patients underwent a general clinical examination, standard 12-lead electrocardiography (ECG), and transthoracic and transesophageal echocardiography (EchoCG). The patients included in the study had functional class (FC) 0–II chronic heart failure (CHF), according to the New York Heart Association (NYHA) functional classification. The exclusion criteria were the following: FC III–IV CHF (NYHA), heart valve disease, as well as systemic, acute, and chronic inflammatory diseases, and cancer.

To assess intracardiac hemodynamic parameters, the patients underwent M-mode and 2D echocardiography from standard positions on the Philips En Visor CHD ultrasound machine (Netherlands). 24 (15.0%) patients had left ventricular hypertrophy, and in 65 patients [61; 67] % of patients, left ventricular ejection fraction was detected.

The patients underwent radiofrequency ablation (RFA), during which myocardial biopsies were taken to exclude viral myocarditis (the apex of the right ventricle, the right ventricular outflow tract and the interventricular septum). AF of unknown etiology was an indication for biopsy. The patients had no complications after the biopsies were taken.

A part of the biopsy sample (1–2 mg) was used to determine the SERCA2a and CASQ2 levels by immunoblotting. The tissue was homogenized (Bullet Blender, Next Advance Inc., USA) in a lysis buffer. Cell membranes were disrupted using ultrasound (Sonopuls, Bandelin). The homogenates were centrifuged

at 16,000g and 4° C for 25 min. The proteins were separated in polyacrylamide gel electrophoresis. Semidry electroblotting (BlueBlot SD, SERVA) was used to transfer proteins onto the nitrocellulose membrane. We used primary SERCA2a (1 : 2,000) and CASQ2 (1 : 2,000) monoclonal antibodies and alkaline phosphatase-conjugated secondary antibodies. BCIP / NBT was used to detect proteins. The level of total protein in the sample was determined on the spectrophotometer at 280 nm corrected for the presence of nucleic acids (260 nm) (NanoVue™, ThermoFisher Scientific). The content of target proteins was calculated relative to the expression of the β-actin protein. All reagents used in the study were manufactured by Sigma-Aldrich (USA).

Genomic DNA was isolated from blood leukocytes of the patients according to the manufacturer's protocol (Promega, USA). The polymorphic variant rs1860561 (110345436G>A in the intron) of the Ca^{2+}ATPase gene (*ATP2A2*) was determined. For the CASQ2 (*CASQ2*) gene, the polymorphic variants rs6684209 (115707991C>T in the intron) and rs7521023 (115700759G>A in the 3′-UTR) were identified. The study was carried out using a real-time polymerase chain reaction (PCR) (DT-96, DNA-Technology, Russian Federation). Primers and fluorescent probes (FAM and HEX) (TestGen, Russian Federation) were used for DNA amplification. The distribution of genotype frequencies was checked for compliance with the Hardy – Weinberg equilibrium using the Pearson's χ2 test.

Statistical analysis was performed using the Statistica 10.0 software (StatSoft Inc., USA). The Shapiro – Wilk test assessed the normality of the sample distribution. Quantitative data were presented as the median and the interquartile range $Me[Q_1; Q_3]$. The differences between the groups were assessed using the Mann – Whitney *U* test. Qualitative data were presented as frequency of occurrence in absolute values and percentages. For qualitative data, the differences between the groups were determined using the Pearson's χ2 or Fisher's exact tests. A cluster analysis identified homogeneous data. The results were considered statistically significant at $p < 0.05$.

RESULTS

Determination of the SERCA2a and CASQ2 proteins in the patients' myocardium showed that the considered sample is heterogeneous in the expression level of these proteins. Thus, the median SERCA2a level in the total sample was 0.667 [0.334; 1.38], and the median CASQ2 level was 0.506 [0.324; 0.858]. Given the great differences in protein expression among the patients, the method of cluster analysis was used to determine possible homogeneous clusters. It resulted in the identification of two clusters significantly different from each other for each protein under study. According to the expression level of SERCA2a and CASQ2, the total sample of patients was subsequently divided into 2 groups: group 1 – patients with a low protein level; group 2 – patients with a high protein level (Fig. 1).

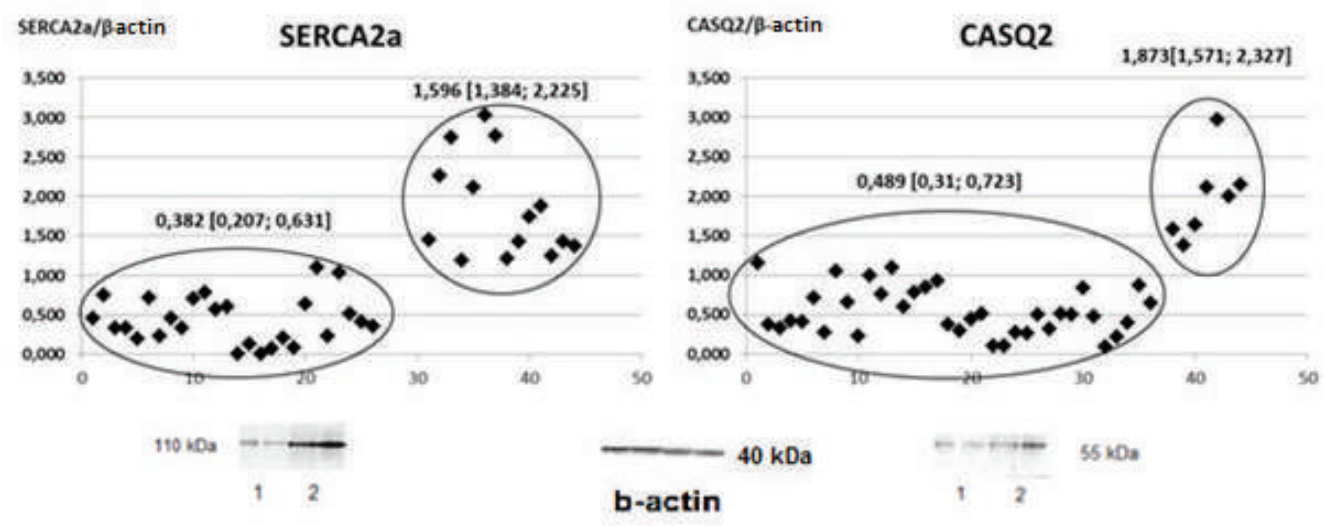

Fig. 1. Clustering of the patients' sample by the expression level of the SERCA2a and CASQ2 proteins

To assess the possible role of the genetic component in the expression of the SERCA2a and CASQ2 proteins in the myocardium of AF patients, we investigated the relationship between the level of these proteins and the presence of polymorphic variants of the *ATP2A2* and *CASQ2* genes. It turned out that all patients were carriers of the polymorphic variant rs1860561 of the *ATP2A2* gene. At the same time, 30 (67%) patients were carriers of the homozygous GG genotype, and 15 (33%) patients were carriers of the heterozygous GA genotype. No patients under study were carriers of the AA genotype.

All patients in the sample were carriers of polymorphic variants rs6684209 and rs7521023 of the *CASQ2* gene. For the polymorphic variant rs6684209, the heterozygous CT genotype was detected in 16 (36%) patients, and the homozygous CC genotype – in 29 (64%) patients. There were no patients with the TT genotype in the study sample. The bulk of the sample with the polymorphic variant rs7521023 (31 patients, 69%) was represented by carriers of the heterozygous genotype AG. 9 (20%) and 5 (11%) patients were carriers of its homozygous genotypes (AA and GG), respectively.

The possible functional significance of each identified genotype was assessed. It turned out (Fig. 2) that in carriers of the homozygous GG genotype of the *ATP2A2* gene, the expression level of the SERCA2a protein was significantly higher (p = 0.039) than in patients with the heterozygous genotype GA and amounted to 0.926 [0.282; 1.65] versus 0.559 [0.123; 1.21], respectively.

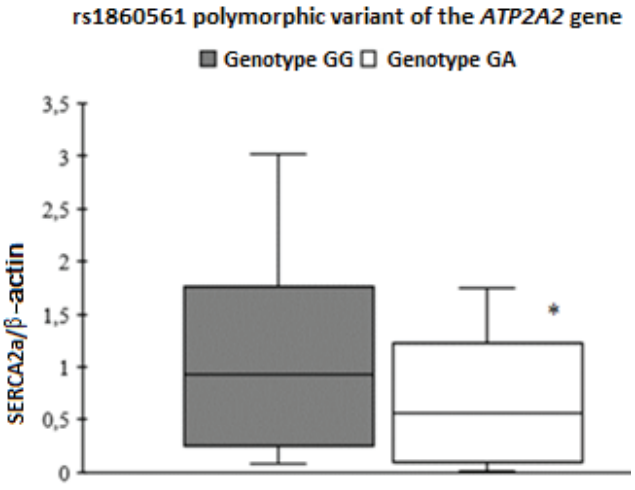

Fig. 2. Expression of the SERCA2a protein depending on the genotypes of the *ATPA2* gene. Here and in Fig. 3: * $p < 0.05$ – statistically significant difference.

The results presented in Fig. 3 show that in carriers of the homozygous CC genotype for the polymorphic variant rs6684209 of the *CASQ2* gene, protein expression was 2.5 times higher than in carriers of the heterozygous genotype and was equal to 0.779 [0.506; 1.380] versus 0.315 [0.272; 0.400], respectively (p = 0.035). The level of protein expression in patients with homozygous genotypes (AA and GG) of the polymorphic variant rs7521023 of the *CASQ2* gene was 0.729 [0.994; 0.517] and 0.516 [2.111; 1.061], respectively, and in patients with the heterozygous genotype AG – 0.479 [0.779; 0.625].

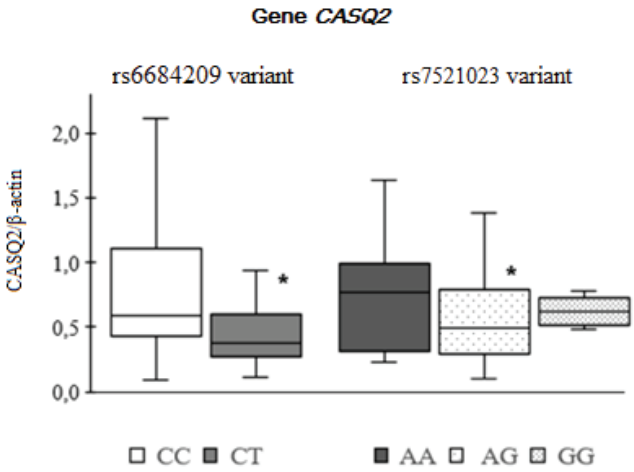

Fig. 3. Expression of the CASQ2 protein depending on the genotypes of the polymorphic variants in the *CASQ2* gene

Analysis of the data on the distribution of patients by clusters depending on the expression level of the SERCA2a protein showed that group 1 with a low level of SERCA2a expression (0.382 [0.207; 0.631]) included 29 patients (64%), and group 2 with a high protein level (1.596 [1.384; 2.225]) consisted of 16 patients (36%). According to the data presented in Table 1, the patients of the first and second groups were comparable in terms of clinical and anamnestic parameters and therapy. However, patients in group 1 were more often prescribed anticoagulants, while patients in group 2 were prescribed antiplatelet drugs.

Table 2 shows the EchoCG results of the patients in the formed groups. Such parameters as the size of the left atrium (LA) and end-systolic and end-diastolic volumes (ESV and EDV) were significantly higher in group 2 than in group 1. At the same time, the rates of early (peak E) and late (peak A) left ventricular diastolic filling were significantly higher in the patients of group 1.

Table 1

Clinical and anamnestic parameters of patients

Parameter	Group 1, $n = 29$	Group 2, $n = 16$	p
Age, years, $Me[Q_1; Q_3]$	45 [40; 51]	42 [38; 48]	0.712
Sex, male / female (n)	19/7	12/2	–
Hypertensive heart disease, n (%)	10 (39)	4 (29)	0.630
Coronary artery disease, n (%)	2 (8)	2 (14)	0.566
Medication			
Statins, n (%)	2 (8)	0	0.300
Antiplatelet agents, n (%)	2 (7)	5 (31)	0.031
Anticoagulants, n (%)	21 (72)	5 (31)	0.008
Antiarrhythmic drugs			
Amiodarone, n (%)	6 (23)	5 (36)	0.544
Allapenin, n (%)	1 (4)	1 (7)	0.902
Propafenone, n (%)	9 (35)	2 (14)	0.269
Sotalol, n (%)	4 (15)	2 (14)	0.915
Bisoprolol, n (%)	3 (12)	0	0.206
Metoprolol, n (%)	2 (8)	2 (14)	0.566

Note: пroup 1 – low level of SERCA2a expression; group 2 – high level of SERCA2a expression.

Table 2

Main structural and functional parameters of the heart in AF patients, *Me* [Q_1; Q_3]

Parameter	Group 1, $n = 29$	Group 2, $n = 16$	p
Ejection fraction, %	65 [62; 68]	63 [60; 66]	0.189
EDV	104 [97; 114]	115 [96; 127]*	0.015
ESV	36 [34; 42]	42 [39; 48]*	0.032
EDD, mm	49 [48; 50]	49.8 [45; 52]	0.902
ESD, mm	31[30; 34]	32 [29; 37]	0.744
LA, mm	37 [35; 42]	42 [39; 45]*	0.035
peak_E, cm / s	82 [72; 88]	69 [62; 80]*	0.039
peak_A, cm / s	61 [59; 66]	47 [44; 52]*	0.018
E / A	1.28 [1.26; 1.5]	1.27 [1.19; 1.55]	0.89
Stroke volume, ml	69.5 [63; 78]	71.5 [59; 79]	0.513
MM, g	174 [157; 186]	173 [138; 211]	0.636
MMI, g / m^2	86.5 [80; 93]	83.5 [71; 95]	0.463

Note: LA – left atrium; ESV – end-systolic volume; EDV – end-diastolic volume; EDD– end-diastolic dimension; ESD – end-systolic dimension; LV – left ventricle; MM – myocardial mass; MMI – myocardial mass index.

A comparative analysis of EchoCG data from patients distributed according to the level of CASQ2 expression in the myocardium did not reveal significant differences between the groups.

DISCUSSION

It is known that the main cause of AF is an abnormal impulse (ectopic activity) [13]. The molecular mechanism of this phenomenon is largely associated with the intracellular homeostasis of calcium ions in cardiomyocytes [14]. Overload of calcium ions in the sarcoplasm of cardiomyocytes leads to a decrease in the electrical stability of the membranes in the cardiac cells and emergence of ectopic pacemakers. Therefore, atrial electrophysiological properties alter, the so-called electrical remodeling occurs [15]. A high atrial rate can stimulate the adaptive response in cardiomyocytes, which is expressed in increased expression of the SERCA2a and CASQ2 proteins.

Thus, the studies by J. Dai et al. found that in AF patients, the expression level of calcium-handling proteins of SR (SERCA2a, phospholamban and ryanodine receptors) in cardiomyocytes was significantly higher than in patients without supraventricular arrhythmias, while the expression level of contractile proteins (troponin T and I, myosin) in the myocardium did not differ [16].

Unfortunately, the available literature does not have any data on the relationship of the polymorphic variants of the *ATP2A2* and *CASQ2* genes with the expression level of their proteins. Our study showed that the presence of specific polymorphic variants and genotypes of the genes of the studied proteins is also significant. It turned out that in carriers of the homozygous GG genotype of the rs1860561 variant of the *ATP2A2* gene and the CC genotype of the rs6684209 variant of the *CASQ2* gene, the level of SERCA2a and CASQ2 expression was significantly higher than in patients with the heterozygous genotype of these genes.

Electrical remodeling can be accompanied by structural changes in the myocardium. In this study, higher SERCA2a expression was associated with greater EDV and ESV values, although these parameters were within the reference values. In this regard, higher EDV values (within the reference values) can be considered as evidence of better maintenance of ventricular diastolic function. This interpretation is quite consistent with the data that SERCA2a overexpression in rabbits with induced AF is accompanied by an increase in the duration of the effective refractory period and an improvement in the myocardial structure [8]. It is possible that excessive hemodynamic load on the atrium in AF adaptively leads to an increase in SERCA2a expression.

This assumption is consistent with the results of our studies, which showed that patients with elevated SERCA2a levels have increased LA size. It is well known that the ventricular filling is divided into two phases: the phase of rapid (active) filling, which is early diastole, and the phase of slow (passive) filling,

corresponding to late diastole, which ends with atrial systole. The phase of rapid filling of the LV characterizes an active relaxation process. At the cellular level at this time, the acto – myosin cross-bridges are disconnected, which is followed by the release of calcium ions into the myoplasm and their reuptake into the SR [17].

Our study showed that patients with a high level of SERCA2a expression had a lower rapid filling rate in early diastole. In addition, these patients had a lower slow filling rate in the late diastole than those with lower SERCA2a expression. The study failed to identify associations of EchoCG parameters of the patients' heart with the level of CASQ2 expression, which may be due to the small number of patients with low CASQ2 expression in our sample.

CONCLUSION

The results of the study allow to conclude that the genotypes of the rs1860561 variants of the *ATP2A2* gene and rs6684209 of the *CASQ2* gene can determine the expression level of SERCA2a and CASQ2. The expression level of SERCA2a is associated with the structural and functional parameters of the heart in AF patients. The obtained results confirm that it is promising to assess the expression level of SERCA2a and CASQ2 to predict the course of cardiovascular pathology and select individual treatment.

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Kondratieva D.S. – determine of the target proteins expression, analyzing and interpreting the data, writing the text of the article. Afanasiev S.A. – develop of the article concept and design, substantiation of the manuscript. Muslimova E.F. – collection of material, determine of the polymorphism of the target genes, data analysis. Archakov E.A. – selection and management of patients, analysis and interpretation of clinical data. Batalov R.E. – analysis of clinical data, verification of critical intellectual content.

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Evaluation of the relationship between nutrition and the risk of fatal outcomes from cardiovascular diseases in people with type 2 diabetes

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ABSTRACT

Aim. To evaluate the relationship between nutrition and the risk of fatal outcomes from cardiovascular diseases in individuals with type 2 diabetes mellitus (T2DM).

Materials and methods. The baseline study was conducted within the HAPIEE project in 2002–2005. The follow-up period for the observed cohort, including individuals with T2DM, lasted from 2003–2005 to December 31, 2018 and reached on average 12.8 years (1.1 ± 16.0 years). Within the study, 2 groups were formed: the treatment group which included persons with T2DM who "developed fatal events" and the control group which encompassed individuals with T2DM who "did not develop fatal events" during the follow-up. The treatment group included 207 people (107 men, 100 women), the average age for both sexes was 62.4 ± 5.9 years; the control group consisted of 474 people (177 men, 297 women), the average age for both sexes was 58.1 ± 6.6 years. The data on actual nutrition were obtained from a survey of the participants using the Food Frequency Questionnaire and included information on consumption of 147 foods. Statistical processing of the data was carried out using the SPSS 13.0 software package. The data were presented as $M \pm SD$, where M is the arithmetic mean and SD is the standard deviation. An analysis of the association between nutrition and the risk of death from cardiovascular diseases was performed using the Cox regression model. The differences were considered statistically significant at $p < 0.05$.

Results. In the studied sample, we identified a significant relationship between the consumption of a number of foods and the risk of death from cardiovascular diseases in people with T2DM over a 15-year follow-up. It was shown that increased consumption of fruits (by 80 g / day) and nuts (by 2 g / day) was significantly associated with a decreased risk of death in men: hazard ratio (HR) = 0.726, $p = 0.044$ and HR = 0.826, $p = 0.011$, respectively. Increased consumption of eggs in men (by 50 g / day) was associated with an increased risk of death: HR = 1.728, $p = 0.003$. In women, a decreased risk of death was observed only with the consumption of meat products: HR = 0.786, $p = 0.036$.

Conclusion. The results of the study showed a clear relationship between the consumption of a number of foods and the risk of death from cardiovascular diseases. Thus, increased consumption of fruits, nuts, and meat products was significantly associated with a decreased risk of death from cardiovascular diseases in people with T2DM, while the consumption of eggs and dairy products, on the contrary, was associated with an increased risk of mortality. These data indicate the importance of dietary control in individuals with carbohydrate metabolism disorders.

Keywords: type 2 diabetes, death from cardiovascular diseases, nutrition, hazard ratio

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Conformity with the principles of ethics. The study was approved by the Ethics Committee at the Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Protocol No. 118a of 17.12.2018).

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Оценка связи питания с риском фатальных исходов от сердечно-сосудистых заболеваний у лиц с сахарным диабетом 2-го типа

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РЕЗЮМЕ

Цель. Изучить связь питания с риском фатальных исходов от сердечно-сосудистых заболеваний у лиц с сахарным диабетом 2-го типа (СД2).

Материалы и методы. Базовое исследование проведено в рамках проекта HAPIEE в 2002–2005 гг. Период наблюдения за наблюдаемой когортой, в том числе за лицами с СД2, длился с 2003–2005 гг. по 31 декабря 2018 г. и составил в среднем 12,8 лет (1,1 ± 16,0 лет). В рамках исследования были сформированы две группы: основная – лица с СД2, у которых «Развились фатальные события», и группа сравнения – лица с СД2, у которых «Не развились фатальные события» за период наблюдения. В основную группу были включены 207 человек (107 мужчин, 100 женщин), средний возраст для лиц обоего пола 62,4 ± 5,9 лет; в группу сравнения – 474 человека (177 мужчины, 297 женщин), средний возраст для лиц обоего пола 58,1 ± 6,6 лет. Данные по фактическому питанию были получены при опросе 681 участника с использованием вопросника по оценке частоты потребления пищевых продуктов и включали информацию о потреблении 147 продуктов. Статистическую обработку данных проводили с использованием пакета прикладных программ SPSS 13.0. Описание данных представлено в виде $M \pm SD$, где M – среднее арифметическое значение, SD – стандартное отклонение. Оценка отношения рисков проведена с использованием регрессии Кокса. Критический уровень статистической значимости различий принимался при $p < 0,05$.

Результаты. Получены данные, что увеличение потребления фруктов (на 80 г/сут) и орехов (на 2 г/сут) связано со снижением рисков сердечно-сосудистой смерти: отношение рисков (HR) = 0,726; $p = 0,044$ и HR = 0,826; $p = 0,011$ соответственно; увеличение потребления яиц (на 50 г/сут) – с повышением риска смерти: HR = 1,728; $p = 0,003$ у мужчин. У женщин при потреблении мясных продуктов наблюдалось снижение риска смерти: HR = 0,786; $p = 0,036$.

Заключение. Результаты исследования показали определенную ассоциацию потребления ряда продуктов с риском смерти от сердечно-сосудистых заболеваний у лиц с СД2. Так, более высокий уровень потребления фруктов, орехов, мясных продуктов был связан со снижением риска сердечно-сосудистой смертности, а увеличение потребления куриных яиц, наоборот, с повышением риска фатального исхода. Эти данные указывают на важность контроля питания у лиц с нарушениями углеводного обмена.

Ключевые слова: сахарный диабет 2-го типа, сердечно-сосудистая смерть, питание, отношение рисков

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

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INTRODUCTION

Currently, cardiovascular diseases (CVDs) are the main cause of death among patients with type 2 diabetes mellitus (T2DM) in the Russian Federation [1]. The traditional risk factors for death in T2DM are gender, age, dyslipidemia, obesity, arterial hypertension (AH), smoking, low physical activity, marital status, and factors associated with the disease itself (the duration of T2DM, the presence of macro- and microvascular complications, hyper- and hypoglycemia) [2, 3]. The inclusion of foods rich in bioactive substances (antioxidants, phenolic acids, isoflavones, anthocyanins, carotenes, unsaturated fatty acids), which improve the parameters of carbohydrate metabolism, in the diet of T2DM patients may be one of the factors for preventing cardiovascular complications [4, 5]. The assessment of the actual nutrition of the population (aged 45–69 years) in Novosibirsk showed that the diet in general, among persons both with and without T2MD, is unbalanced and does not correspond to the recommendations [6].

The aim of the study was to investigate the relationship between nutrition and the risk of fatal outcomes from cardiovascular diseases in people with T2MD.

MATERIALS AND METHODS

The research was carried out on the material of the Russian branch of the international HAPIEE (Health, Alcohol and Psychosocial factors In Eastern Europe) study "Determinants of Cardiovascular Diseases in Eastern Europe". The object of the HAPIEE study was a population sample including residents (aged 45–69 years) of two administrative districts of Novosibirsk, typical both of Novosibirsk and other large industrial cities of Siberia. The sample was formed on the basis of the electoral register using random number tables. The total sample size from the general population was determined by the protocol of the HAPIEE project. From 2003 to 2005, the staff of the Research Institute of Internal Medicine of the Siberian Branch of the Russian Academy of Medical Sciences (since 2017 – Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences) examined 9,360 men and women of the specified age (the principal researchers – the Academician Yu. P. Nikitin and Prof. S. K. Malyutina). The response made 61% [7].

Out of 9,360 examined individuals, T2DM was detected in 982 people (for the first time, during screening and before screening). In total, 301 people were excluded from the study: persons with T2DM who reported nonfatal myocardial infarction and / or stroke in their medical history during the baseline survey (139 people), persons whose cause of death was not CVD during the follow-up (123 people), and 39 people about whom no information was received during the follow-up. Thus, the analysis included the data from the baseline survey of 681 people with T2DM (284 men, 397 women).

The follow-up period for the observed cohort, including persons with T2DM, lasted from 2003–2005 to December 31, 2018 and made on average 12.8 years (1.1 ± 16.0 years). Fatal outcomes in the study cohort were identified by combining several sources of information. We copied the data from the Medical Certificates of Cause of Death for the period from 01.02.2003 to 31.12.2018, obtained at the Civil Registry Office in the city of Novosibirsk. Information about fatal events was also collected during repeat screenings in 2006–2008 and 2015–2017 and two postal surveys. The causes of overall and cardiovascular (CV) death were established in accordance with the codes of the International Classification of Diseases, 10th revision

(ICD-10). Cardiovascular death was established according to the ICD codes I (0–99).

Within the study, 2 groups were formed: the treatment group which included persons with T2DM who "developed fatal events" – 207 people (107 men, 100 women) and the control group which encompassed individuals with T2DM who "did not develop fatal events" during the follow-up – 474 people (177 men and 297 women).

All project participants underwent a baseline screening, which included anthropometric measurements (height, weight, body mass index calculation) and collection of information using the structured questionnaire of the HAPIEE project on the presence of T2DM and its duration, as well as on the presence of AH, education, marital status, smoking status, and the level of physical activity. To assess actual nutrition, the adapted Food Frequency Questionnaire was used to determine the frequency of food consumption [8]. 147 products were included in the questionnaire. Nutrition was assessed over the previous 3 months according to answers to 9 questions on the frequency of consumption of a certain product. The answers ranged from "never or less than once a month" to "up to six or more times a day". The survey was conducted by a trained interviewer [9].

Statistical processing of the data was carried out using the SPSS v.13.0 software package. The data were presented as $M \pm SD$, where M is the arithmetic mean, and SD is the standard deviation. Hazard ratio (HR) was calculated using the Cox regression model. The differences were considered statistically significant at $p < 0.05$.

RESULTS

The men in the treatment group were 4 years older than in the control group (60.78 ± 6.52 vs. 56.88 ± 6.53 years; $p < 0.001$), and the women in the treatment group were 5 years older than in the control group (64.15 ± 6.14 vs. 58.81 ± 6.13 years; $p < 0.001$). Body mass index (BMI) among men (29.55 ± 4.86 vs. 29.66 ± 4.88 kg / m^2; $p = 0.857$) and women (33.81 ± 5.56 vs. 32.62 ± 5.51 kg / m^2; $p = 0.064$) did not differ significantly between the groups. The total energy intake from nutrition both among men (2,562 ± 799 vs. 2,777 ± 803 kcal / day; $p = 0.030$) and women (2,084 ± 633 vs 2,262 ± 632 kcal / day; $p = 0.016$) was lower in the treatment group.

Table 1 presents the data on food consumption (g / day) among the residents of Novosibirsk (aged 45–69 years) with T2DM (baseline screening within the HAPIEE study, $M \pm SD$) who developed / did not develop fatal cardiovascular events during the follow-up. The men from the treatment group consumed less fruits and nuts and more chicken eggs and dairy products than those in the control group. For the rest of the products, no significant difference in consumption was revealed. Increased consumption of white bread and decreased consumption of meat products were observed among the women of the treatment group.

Table 2 shows the results of the Cox regression analysis on the relationship between the risk factors (consumption of the studied products, g / day) and death from CVD among the people with T2DM, adjusted for age, BMI, total energy intake, educational level, marital status, smoking status, AH, and the level of physical activity. Using this method, we estimated the hazard ratio (HR) of fatal CV event development with an increase in individual food item consumption calculated by a certain amount (g / day) [10]. It was found that the risk of a fatal CV event among men decreased by 27.4% with an increase in the consumption of some fruits (by 80 g / day) ($p = 0.044$). The "fruits" group included apples, pears, oranges, grapefruits, tangerines, peaches, apricots, and bananas. The risk of a fatal CV event among men also decreased by 17.4% with an increase in the consumption of nuts (by 2 g / day) ($p = 0.011$). The increased consumption of meat products (by 80 g / day) reduced the risk of fatal CV events among women by 21.4% ($p = 0.036$).

An increase in the consumption of chicken eggs by men (by 50 g / day – one more egg on average) increased the risk of CV death by 1.7 times ($p = 0.003$). The consumption of dairy products (excluding milk) increased the risk of CV death by 1.1 times ($p = 0.029$). No such correlations were observed for women (Table 2). Previously, the authors presented the relationships of such factors as AH, abdominal obesity, low level of physical activity, current smoking, and marital status with CV mortality among people with T2DM living in Novosibirsk [3].

DISCUSSION

The results of the study showed that the increased consumption of fruits and nuts was associated with a decreased risk of death from CVD among men. The data obtained are consistent with the results of a number of population-based studies. The article by G. Liu et al. (2019) presents the results of two prospective, cohort studies that included 16,217 men and women with T2DM, which showed that the consumption of nuts (the consumption was more than 20 g / day in the

highest quintile and less than 0.9 g / day in the lowest quintile) was associated with significant reduction of a risk for CVD death (the relative risk in the highest quintile compared with the lowest one was HR = 0.66; 95% confidence interval (CI) 0.52–0.84). The effect was greater with the consumption of hazelnuts (walnuts, almonds, Brazil nuts, cashews, pistachios, cobnuts, pine nuts) and peanuts (legumes).

As the authors of the work note, the mechanism of the positive effects of nut consumption among the persons with T2DM is currently not clear [11], although this can be primarily determined by the rich nutritional composition of nuts (polyunsaturated fatty acids, dietary fiber, vitamins, minerals (calcium, potassium, magnesium), and phytochemicals (flavonoids, phytosterols)) [12]. The study on the relationship between the consumption of fruits, vegetables, and legumes in the diet of Europeans with T2DM (10,449 participants) and fatal outcomes from CVDs showed a significant decrease in the relative risk of death with increased consumption of legumes (RR = 0.72; CI 0.60–0.88) and fruits (relative risk (RR) = 0.90; 95% CI 0.81–0.99) (adjusted for gender, age, total energy intake, smoking status, history of heart diseases, cancer, and AH). At the same time, no significant relationship with vegetable consumption was revealed (RR = 0.85; 95% CI 0.85–1.07) [10].

The data from a number of prospective meta-analyses also show the positive impact of fruits and nuts. Thus, the meta-analysis of 15 prospective studies showed that the increased consumption of fruits and vegetables was associated with a 10% decrease in the all-cause mortality (RR = 0.90, CI 0.87–0.93) [13]. The analysis of 17 studies on the consumption of vegetables and nuts found that nut consumption was associated with a reduced risk of death (all nuts: RR = 0.78, 95% CI 0.72–0.84; hazelnuts: RR = 0.82, 95% CI 0.75–0.90; peanuts: RR = 0.77, 95% CI 0.69–0.86) [14]. The meta-analysis (15 studies) on the consumption of nuts [15] showed 20% reduction of a risk of all-cause mortality (consumption of nuts: RR = 0.81, 95% CI 0.77–0.85; hazelnuts: RR = 0.80, 95% CI 0.74–0.86; peanuts: RR = 0.85, 95% CI 0.82–0.89), which indicates a positive effect of the increase in the consumption of these products in the diet.

In our study, we obtained the data that the increase in the meat product consumption (by 80 g / day) reduced the risk of a fatal CV event among women with T2DM. A number of studies including a big population sample showed a significant increase in the risk of all-cause mortality and mortality from CVDs with the consumption of red meat and processed meat products (sausages, ham, canned meat) [16, 17]. However, it was also shown that the consumption of meat products which included chicken, turkey, fish, poultry cuts, lean sausages, and poultry hot dogs was associated with the decrease in the all-cause mortality and mortality from CVDs [17]. It was noted that the increase in the proportion of animal protein in relation to plant protein was associated with an increased risk of death, also among the persons with T2D [18]. In a large, prospective cohort study of Japanese residents (70,696 participants aged 45–74 years), higher consumption of plant protein was associated with a lower risk of all-cause mortality and death from CVDs [19].

Increased egg consumption was associated with an increased risk of death from CVDs among men. The association between egg consumption and the risk of death is supported by the results of a prospective cohort study in the United States (21,327 participants aged 40–86 years), which found the relationship of chicken egg consumption with the relative risk of all-cause mortality when comparing the highest quintile of consumption (≥ 7 eggs/ week) with the controls (< 1 egg / week) (HR = 1.22; 95% CI 1.09–1.35); among the people with T2DM, the risk of death was 2 times higher (HR = 2.01; 95% CI 1.26–3.20) [20]. In another study of US residents (29,615 participants), higher egg consumption was associated with an increased risk of all-cause mortality (by 8%) (HR = 1.08; 95% CI 1.04–1.11) [21].

Our analysis also showed that the increase in the consumption of dairy products (sour cream, kefir, dairy desserts, cottage cheese, and cheese) by 50 g / day ($p = 0.029$) increased the risk of fatal CVDs by 1.1 times among men ($p = 0.029$), which requires further research.

CONCLUSION

The results of the study indicate a certain role of the consumption of particular foods as an “unconventional” factor in the development of a fatal CV event among persons with T2DM, when taking into account traditional risk factors, such as age, BMI, total energy intake, smoking status, educational level, marital status, AH, and the level of physical activity. It can be noted that there are differences between men and women. While the observed relationships between fruit and nut consumption and the decrease in the risk of death from CVD, as well as between the increase in the consumption of eggs and the increased risk of death from CVDs among men comply with the find-

ings of current population-based studies, the relationship between the consumption of meat products and the decrease in the risk of death from CVDs among women requires a more thorough analysis.

The complexity of evaluating the relationship between the consumption of meat products and the risk of death is determined by the difference and diversity of the consumed meat products (beef, lamb, pork, sausages, chicken, rabbit meat, offal, ham, loin, pates, canned meat), the method of meat preparation (boiled, fried, smoked, canned, salted), and the plant protein / animal protein ratio. Evaluation of the relationship of dairy products with the risk of fatal outcomes among men also requires further research because of the weakly expressed association.

It should be noted that the average consumption of nuts was almost 4 times lower than the recommended level. Since there are no definite recommendations for the inclusion of nuts in the diet of people with T2DM, the results obtained indicate their potential positive role in mortality reduction among people with carbohydrate metabolism disorders and should be taken into account when developing and updating dietary recommendations.

Table 1

Data on nutrition of Novosibirsk residents (aged 45–69 years) with T2DM (baseline screening within the HAPIEE study, *M* ± *SD*) who developed / did not develop fatal cardiovascular events during the follow-up (men and women – adjustment for age, BMI, and total energy intake; both genders – adjustment for gender, age, BMI, and total energy intake)

Food groups, g / day	Both genders		*p*	Men		*p*	Women		*p*
	Fatal cardiovascular events have developed, *n* = 207	Fatal cardiovascular events have not developed, *n* = 474		Fatal cardiovascular events have developed, *n* = 107	Fatal cardiovascular events have not developed, *n* = 177		Fatal cardiovascular events have developed, *n* = 100	Fatal cardiovascular events have not developed, *n* = 297	
Fruits	78.76±86.40	99.38±83.78	0.006	63.59±75.38	90.53±74.65	0.005	99.56±93.08	111.31±89.61	0.284
Vegetables	259.63±164.9	281.64±159.8	0.132	246.15±138.2	271.66±136.8	0.141	281.26±183.9	298.06±177.1	0.438
Cereals	79.31±55.89	79.88±54.38	0.906	79.15±50.56	79.61±50.12	0.942	80.82±60.90	81.55±58.58	0.919
Legumes	12.37±12.58	12.04±12.27	0.767	10.82±10.89	10.52±10.78	0.822	14.15±14.01	13.82±13.78	0.837
Nuts	2.51±8.91	4.95±8.68	0.002	2.19 ± 10.68	5.82±10.58	0.007	3.22±7.53	4.33±7.25	0.212
White bread	72.75±50.98	62.36±49.66	0.019	85.83±53.60	80.20±53.06	0.401	60.96±50.04	46.47±48.17	0.014
Black bread	40.89±51.23	46.40±45.35	0.173	42.61±48.60	44.42±48.10	0.766	39.19±46.07	48.46±44.37	0.088
Sweets	79.01±55.54	81.00±53.97	0.667	88.21±59.97	91.67±59.35	0.645	75.55±52.52	75.62±50.57	0.991
Meat products	176.64±69.34	190.95±67.31	0.017	205.97±79.10	219.94±78.29	0.158	152.24±60.73	168.09±58.48	0.027
Fish	38.41±27.30	39.70±26.47	0.581	39.97±29.06	41.29±28.77	0.715	38.11±26.37	39.24±25.40	0.717
Eggs	20.40±19.80	15.50±19.21	0.004	27.11±25.49	19.37±25.19	0.016	13.90±14.34	12.29±13.80	0.339
Dairy products	143.23±119.4	121.66±115.7	0.036	152.40±129.0	114.94±127.7	0.021	138.68±113.4	131.68±109.2	0.600
Milk	127.68±166.9	107.22±161.5	0.154	146.56±199.6	123.72±201.6	0.365	112.21±138.0	93.59±132.8	0.252
Fats, oil	36.30±15.97	33.12±15.59	0.021	37.08±17.26	33.68±17.08	0.117	36.25±15.01	33.50±14.46	0.120

Table 2

Results of the Cox regression analysis on the relationship between the risk factors (consumption of the studied products) and fatal outcomes from CVD among people with T2DM (* adjustment for age, BMI, total energy intake, educational level, marital status, smoking status, arterial hypertension, and physical activity)

Food groups, g / day	Both genders, *n* = 681	Men, *n* = 284	Women, *n* = 397
Fruits (by 80 g / day)			
HR	0.786 (*p* = 0.011)	0.726 (*p* = 0.044)	0.852 (*p* = 0.228)
95% CI	0.670–0.923	0.570–0.990	0.670–1.083
Vegetables (by 80 g / day)			
HR	0.923 (*p* = 0.220)	1.000 (*p* = 0.571)	0.923 (*p* = 0.299)
95% CI	0.852–1.000	0.852–1.083	0.852–1.083
Cereals (by 50 g / day)			
HR	1.000 (*p* = 0.821)	1.051 (*p* = 0.662)	1.000 (*p* = 0.965)
95% CI	0.905–1.162	0.860–1.283	0.818–1.221
Legumes (by 10 g / day)			
HR	1.030 (*p* = 0.624)	1.127 (*p* = 0.305)	0.990 (*p* = 0.944)
95% CI	0.904–1.184	0.904–1.397	0.834–1.184
Nuts (by 2 g / day)			
HR	0.895 (*p* = 0.006)	0.826 (*p* = 0.011)	0.939 (*p* = 0.178)

Table 2 (continued)

Food groups, g / day	Both genders, *n* = 681	Men, *n* = 284	Women, *n* = 397
95% CI	0.828–0.968	0.662–0.956	0.857–1.028
White bread (by 50 g / day)			
HR	1.105 (*p* = 0.234)	1.000 (*p* = 0.874)	1.221 (*p* = 0.060)
95% CI	0.951–1.283	0.818–1.221	1.000–1.489
Black bread (by 50 g / day)			
HR	0.951 (*p* = 0.601)	1.105 (*p* = 0.372)	0.778 (*p* = 0.054)
95% CI	0.818–1.105	0.905–1.349	0.605–1.000
Sweets (by 50 g / day)			
HR	1.000 (*p* = 0.739)	0.951 (*p* = 0.707)	1.000 (*p* = 0.840)
95% CI	0.860–1.105	0.818–1.162	0.778–1.221
Meat products (by 80 g / day)			
HR	0.852 (*p* = 0.083)	0.923 (*p* = 0.318)	0.786 (*p* = 0.036)
95% CI	0.726–1.000	0.726–1.083	0.618–0.990
Fish (by 30 g / day)			
HR	1.000 (*p* = 0.938)	1.000 (*p* = 0.938)	0.942 (*p* = 0.671)
95% CI	0.835–1.196	0.786–1.270	0.696–1.270
Eggs (by 50 g / day)			
HR	1.728 (*p* = 0.001)	1.728 (*p* = 0.003)	1.815 (*p* = 0.097)
95% CI	1.283–2.223	1.221–2.440	0.905–3.437
Dairy products (by 50 g / day)			
HR	1.051 (*p* = 0.036)	1.051 (*p* = 0.029)	1.051 (*p* = 0.537)
95% CI	1.000–1.105	1.000–1.162	0.951–1.105
Milk (by 80 g / day)			
HR	1.000 (*p* = 0.523)	1.000 (*p* = 0.789)	1.083 (*p* = 0.431)
95% CI	0.923–1.083	0.923–1,083	0.923—1.173
Fats, oil (by 15 g / day)			
HR	1.094 (*p* = 0.197)	1.078 (*p* = 0.438)	1.178 (*p* = 0.150)
95% CI	0.956–1.250	0.900–1.288	0.942–1.491

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Authors contribution

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Evaluation of the functional state of mitochondria isolated from mononuclear leukocytes by flow cytometry in patients with chronic heart failure receiving ubidecarenone

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ABSTRACT

Aim. To evaluate the functional state of mitochondria isolated from peripheral blood mononuclear leukocytes using flow cytometry in patients with chronic heart failure receiving ubidecarenone (coenzyme Q).

Materials and methods. The study included 53 patients with chronic heart failure who had experienced myocardial infarction. The patients were divided into two groups: group 1 received optimally chosen standard therapy, while group 2 received optimally chosen standard therapy and ubidecarenone ("Kudevite"). The mitochondrial membrane potential was evaluated by flow cytometry using propidium iodide and 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)). The levels of coenzyme Q were determined using high-performance liquid chromatography with ultraviolet (UV) detection.

Results. A direct correlation was established between the coenzyme Q levels in the blood plasma and the percentage of DiOC6(3)-positive cells ($R = 0.39$; $p < 0.05$) in the patients with chronic heart failure. In group 1, no significant differences in the coenzyme *Q* levels and the percentage of DiOC6(3)-positive and DiOC6(3)-negative cells before and after the therapy were observed. In group 2, a significant increase in the proportion of DiOC6(3)-positive cells and a significant decrease in the percentage of DiOC6(3)-negative cells were revealed.

Conclusion. The increase in the functional activity of mitochondria in the patients with chronic heart failure receiving ubidecarenone was identified. Flow cytometry can be used to evaluate the functional state of mitochondria and observe the efficiency of the selected therapy.

Keywords: mitochondria, chronic heart failure, coenzyme Q, flow cytometry, mitochondrial membrane potential

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Оценка функционального состояния митохондрий мононуклеарных лейкоцитов методом проточной цитометрии у пациентов с хронической сердечной недостаточностью под влиянием убидекаренона

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РЕЗЮМЕ

Цель – оценить функциональное состояние митохондрий мононуклеарных лейкоцитов периферической крови с применением метода проточной цитометрии у пациентов с хронической сердечной недостаточностью на фоне приема препарата убидекаренона (коэнзима Q).

Материалы и методы. В исследование включены 53 пациента с хронической сердечной недостаточностью после перенесенного инфаркта миокарда. Пациенты были распределены в две группы: первая группа получала только оптимально подобранную стандартную терапию, вторая группа – дополнительно к оптимально подобранной медикаментозной терапии получала препарат убидекаренона («Кудевита»). Оценка митохондриального мембранного потенциала проводилась методом проточной цитометрии с применением йодистого пропидия и йодид 3,3'-дигексилоксакарбоцианина (DiOC6(3)). Определение содержания коэнзима Q в крови проводилось методом высокоэффективной жидкостной хроматографии с ультрафиолетовой детекцией.

Результаты. Выявлена прямая корреляционная зависимость между содержанием коэнзима Q в плазме крови и процентом DiOC-позитивных клеток (R = 0,39; p < 0,05) у пациентов с хронической сердечной недостаточностью. В группе пациентов, получавших только оптимально подобранную стандартную терапию, не выявлено статистически значимых различий в содержании коэнзима Q и процентном содержании DiOC-позитивных и DiOC-негативных клеток до начала и после терапии. В группе пациентов, получавших дополнительно препарат убидекаренона, после терапии наблюдалось статистически значимое увеличение доли DiOC-позитивных клеток и уменьшение доли DiOC-негативных клеток.

Заключение. Установлено повышение функциональной активности митохондрий у пациентов с хронической сердечной недостаточностью на фоне терапии препаратом убидекаренона. Метод проточной цитометрии может быть использован для оценки функционального состояния митохондрий и контроля эффективности применяемой терапии.

Ключевые слова: митохондрии, хроническая сердечная недостаточность, коэнзим Q, проточная цитометрия, митохондриальный мембранный потенциал

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

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INTRODUCTION

The use of ultrastructural analysis to study pathological processes in the myocardium has shown that mitochondria are the first to respond to any impact [1]. Identifying the nature and degree of mitochondrial damage in patients with chronic heart failure (CHF) is an important clinical and laboratory task, as it can determine the severity of the disease course [2, 3]. In addition, mitochondria are seen as a potential target for heart failure therapy [4–6].

To assess the functional state of mitochondria (M), there is a fairly large set of methods based on measuring the rate of oxygen uptake, autofluorescence of NADH and flavoproteins, the activity of M enzymes, and ATP levels [7]. However, not all methods can be used in clinical diagnostic laboratories. First of all, this is due to high complexity of research algorithms and analysis methods. One of the promising methods for studying the functional state of M is the assessment of changes in the mitochondrial membrane potential (MMP) using flow cytometry. The advantages of the method include a small amount of biological material required for the study and higher throughput capacity.

A drop in MMP can serve as an integral indicator of the functional state of M, since one of the most important functions of M is energy supply of cells, in which the respiratory chain plays an important role. The activity of the respiratory chain is accompanied by release of protons into the intermembrane space, which leads to the formation of a proton gradient, which triggers the activity of ATP synthase. Metabolic disorders, as well as the structure and integrity of the mitochondrial membrane, can ultimately result in a decrease in the MMP [8, 9].

Determination of MMP changes using flow cytometry is based on the use of special fluorescent dyes. Examples of such dyes are 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) and propidium iodide (PI) [10, 11]. DiOC6(3) belongs to the group of lipophilic cationic dyes, which are called "mitochondrial probes" in the literature. Due to its lipophilic properties, DiOC6(3) is able to freely penetrate into the bilipid membranes of the cell, and, due to its cationic properties, this dye accumulates in areas with a high concentration of protons, that is, in mitochondria. This effect is accompanied by a change in the intensity of cellular fluorescence in the green region of the spectrum, which is recorded by flow cytometry [12]. If the proton concentration in the M is reduced, the dye will accumulate in them less efficiently, and, as a consequence, the intensity of its fluorescence will decrease. Thus, it is possible to distinguish between cells with efficiently functioning M and, consequently, high fluorescence intensity (DiOC6(3)-positive cells) and cells in which the functioning of M is impaired (DiOC6(3)-negative cells). Such cells have reduced fluorescence intensity.

In later stages of cell destruction, the integrity of the cell membrane is disrupted and, as a result, cell death occurs. To detect these late stages, another fluorescent dye, PI, is used, which cannot penetrate through cell membranes, but as it degrades, it begins to penetrate into the cell, accumulating in the cytoplasm and nucleus and interacting with DNA and RNA. As a result, the cell acquires the ability to fluoresce in the red region of the spectrum.

Thus, the method of flow cytometry using two fluorescent dyes makes it possible to detect not only cells with preserved functional state M, but also to identify cells at different stages of apoptosis, which is a consequence of developing mitochondrial dysfunction [13].

Given the complexity of obtaining human cardiac tissue for research purposes, one of the approaches for studying pathogenetic changes in the myocardium is to determine biochemical parameters in peripheral blood cells. The literature contains evidence of a correlation between changes in internal organs, including the myocardium, and changes in peripheral blood cells [14–16]. Thus, the works by E. Cortez et al. showed a correlation between changes in the biochemical parameters in peripheral blood mononuclear leukocytes and cardiomyocytes. The following parameters were determined: cellular respiration rate, carnitine palmitoyltransferase I, UCP 2, GLUT 1 [14].

The aim of the study was to assess the functional state of M of peripheral blood mononuclear leukocytes using flow cytometry in patients with CHF against the background of ubidecarenone (coenzyme Q) administration.

The objectives of the study were to determine the possibility of evaluating the effectiveness of ubidecarenone therapy by monitoring changes in the MMP in blood cells in CHF patients.

MATERIALS AND METHODS

The study included 53 patients with CHF who experienced myocardial infarction (MI) in the last 6 months before inclusion in the study. The patients were treated at St. Petersburg State Government-Funded Healthcare Institution "Elizavetinskaya Hospital". The study was conducted in accordance with the Declaration of Helsinki "Recommendations Guiding Physicians in Biomedical Research Involving Human

Subjects" and the requirements outlined in the main regulatory documents on clinical trials in the Russian Federation. The average age of the patients was 68 ± 8.1 years, including 28 men and 25 women. The diagnosis of CHF was based on the criteria of the Society of Heart Failure Specialists.

All patients were initially evaluated for the functional state of M of peripheral blood mononuclear leukocytes and the content of total coenzyme Q (CoQ) in the blood plasma. Besides, standard clinical and biochemical examinations were performed. The patients were then divided into two groups by block randomization (2 × 2). Group 1 included 28 patients who received optimally chosen standard therapy in accordance with the clinical guidelines for the diagnosis and treatment of coronary artery disease (CAD) and CHF. Group 2 encompassed 25 patients who received optimally chosen standard therapy and ubidecarenone ("Kudevite") at a dose of 120 mg per day (2 capsules (30 mg per capsule) in the morning and 2 capsules in the evening).

Patients of groups 1 and 2 were comparable in gender and age: the average age in group 1 was 70.0 ± 6.9 (56.0; 78.0) years, in group 2 – 66.8 ± 9.5 (49.0; 78.0) years ($p > 0.05$). In group 1, men accounted for 48%, in group 2 – for 52%. All studies were carried out twice: at hospitalization and 3 months after the start of the therapy.

To assess changes in MMP by flow cytometry, blood was collected in tubes containing EDTA. The leukocyte suspension was isolated using urografin. The obtained supernatant was used for the study. Then, a 20-fold DiOC6(3) solution (Invitrogen, USA) was added to 100 μl of the cell suspension, resulting in a final concentration of DiOC6(3) equal to 20 nM. The samples were then thoroughly mixed and incubated for 20 minutes at 37 °C in a 5% CO2 atmosphere in a dark place. The resulting cell suspension was added to 10 μl of a propidium iodide (PI) solution (Sigma-Aldrich, USA), obtaining a final concentration of PI equal to 1 μg / ml. The samples were then incubated for 10 minutes at room temperature in the dark place. Upon completion of the incubation, 200 μl of phosphate-buffered saline was added to the samples, and flow cytometric counting was performed. The obtained results were analyzed using the Kaluza ™ software (Beckman Coulter, USA).

The total plasma CoQ was determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection on the Agilent 1200 series gradient system [17, 18]. CoQ determined in the blood plasma of group 1 patients before the start of the treatment was endogenous CoQ. In the patients of group 2, the determined CoQ was the sum of the endogenous CoQ and that obtained together with the drug.

As a drug containing ubidecarenone (CoQ), the drug "Kudevite" (PIK-PHARMA, Moscow) was used in the study. A feature of this pharmaceutical preparation is a high-tech substance ALL-Q produced in Switzerland, which provides optimal bioavailability of ubidecarenone. This substance has increased hydrophilicity, which makes it possible to convert poorly absorbed hydrophobic ubidecarenone into a water-soluble form that is optimal for absorption.

The research materials were statistically processed using parametric and nonparametric tests. Accumulation, correction, and systematization of the baseline data and visualization of the results were carried out in Microsoft Office Excel 2016 spreadsheets. Statistical analysis was carried out using the STATISTICA 10 software (StatSoft.Inc., USA).

Aggregates of quantitative variables with non-normal distribution were presented as the median and the interquartile range Me (Q_1–Q_3). To compare aggregates of independent variables with non-normal distribution, the Mann – Whitney U-test was used. In order to study the relationship between the quantitative data, the Spearman's rank correlation coefficient was used. The values of the correlation coefficient ρ were interpreted in accordance with the Chaddock scale (Table 1).

Table 1

Chaddock scale	
The values of the correlation coefficient r_{xy}	Characteristics of the strength of the correlation
less than 0.1	no correlation
0.1–0.3	weak
0.3–0.5	moderate
0.5–0.7	noticeable
0.7–0.9	high
0.9–0.99	very high

The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The correlation analysis revealed a significant moderate positive correlation between the content of endogenous CoQ in the blood plasma (0.55 ± 0.11 μg / ml) and the percentage of DiOC6(3)-positive cells ($R = 0.39$, $p < 0.05$) in patients with CHF before the treatment. Low concentration of CoQ in patients with CHF is one of the factors leading to changes in mitochondrial processes and activation of processes

leading to cell death, which results in a decrease in the percentage of DiOC6(3)-positive cells where the functioning of M is preserved. The higher the concentration of CoQ, the more stable the mitochondrial membrane. Thus, the addition of ubidecarenone to therapy can improve the functional state of M.

In group 1 (patients who received only optimally chosen standard therapy), no statistically significant differences in the percentage of cells before and after the therapy were revealed (Table 2). In group 2 (patients who additionally received ubidecarenone), a statistically significant increase in the percentage of DiOC6(3)-positive cells and a decrease in the percentage of DiOC6(3)-negative cells were observed after the therapy.

Table 2

The content of DiOC6(3)-positive and DiOC6(3)-negative cells in patients with CHF before and after the treatment, %, *Me* (Q_1–Q_3)

Parameter	DiOC6(3)-positive cells	DiOC6(3)-negative cells, %
Group 0 (before the start of the treatment)	75.0 (67.0; 80.4)	21.5 (19.5; 32.9)
Group 1 (standard therapy)	77.0 (71.0; 85.4)	21.8 (14.4; 28.9)
Group 2 (standard therapy + “Kudevite”)	94.0 (80.0; 95.0)*	4.2 (4.0; 19.5)**

* $p = 0.025$ (when compared with group 0), $p = 0.044$ (when compared with group 1).
** $p = 0.031$ (when compared with group 0), $p = 0.043$ (when compared with group 1).

The correlation analysis in group 2 showed a significant negative correlation between the content of CoQ in the blood plasma and the baseline percentage of DiOC6(3)-negative cells ($R = – 0.45$, $p < 0.05$).

DiOC6(3)-positive cells exhibit high fluorescence in the green region of the spectrum, which is associated with active accumulation of the DiOC6(3) dye. This indicates preservation of MMP and, consequently, the main processes aimed at its formation. Thus, against the background of ubidecarenone therapy, an increase in the functional activity of M is observed. It is known that ubidecarenone, penetrating into cells, is involved in the work of the respiratory chain and participates in cellular energy supply [19, 20]. In addition, ubidecarenone has antioxidant properties and reduces production of reactive oxygen species (ROS). ROS, through the activation of MAP kinases, including p38 and p53, activate proapoptotic factors (Bax, Bak, and others) and contribute to opening of mitochondrial pores. A drop in ROS production leads to a decrease in the release of proapoptotic proteins from the mitochondrial matrix into the cytosol, suppression of apoptosis, and, as a consequence, a decrease in subsequent cell death. Thus, the functional activity of M, including the work of the respiratory chain, is preserved. As a result, the release of protons into the intermembrane space is restored, leading to restoration of the MMP.

Against the background of ubidecarenone therapy, a decrease in the percentage of DiOC6(3)-negative cells was observed, which may be associated with a decrease in ROS production and suppression of apoptosis intensity. Due to the increased concentration of protons in the intermembrane space of M, DiOC6(3) is more intensively accumulated in M, leading to an increase in the fluorescence intensity in the green region of the spectrum. At the same time, due to the preservation of the cell membrane structure following a decrease in the activity of lipid peroxidation, penetration of another dye (PI) into cells and its accumulation in them decreases. This is manifested through a decrease in the fluorescence intensity in the red region of the spectrum, which indicates a decrease in the number of cells at the stage of early apoptosis and dying cells.

CONCLUSION

Thus, flow cytometry revealed an increase in the functional activity of M in patients with CHF during ubidecarenone therapy, which is confirmed by a significant increase in the percentage of DiOC6(3)-positive cells. Determination of the functional state of M of peripheral blood mononuclear leukocytes using flow cytometry can be used to assess the functional state of M and monitor the effectiveness of therapy in patients with CHF.

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Authors contribution

Lobanova O.A., Ermakov A.I. – carrying out of the research, analysis and interpretation of the data. Gaykovaya L.B. – conception and design. Dadali V.A. – final approval of the manuscript for publication. Kukharchik G.A. – selection of patients.

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Morphological analysis of a local tissue response to subcutaneously implanted acellular dermal matrix fragments

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ABSTRACT

Acellular dermal matrices (ADMs) are gaining popularity as surgical materials for operations on the pelvic organs, as well as in burn therapy and plastic surgery. Evaluation of the biocompatibility of surgical materials is an important and necessary step in the development of new ADMs.

The aim of the study was to compare the results of subcutaneous implantation of ADM and native porcine skin in rats.

Materials and methods. To obtain ADMs, detergent – enzymatic decellularization was used. On days 7, 14, 21, and 60 after the implantation of ADMs (the experimental group) and native porcine skin (the control group), the animals were removed from the experiment. The histologic sections were stained with hematoxylin – eosin and Masson's trichrome stain, then an immunohistochemical reaction with antibodies to CD3 and CD68 was performed. Computer morphometry was carried out using the ImageJ software.

Results. On day 7 after the implantation, moderate sterile inflammation in the experimental group and pronounced sterile inflammation with eosinophil infiltration in the control group were observed. On day 14 of the experiment, the samples from the experimental group were characterized by a relatively low content of macrophages and T-lymphocytes with insignificant edema and no signs of ADM biodegradation. The control group showed pronounced inflammation, a large number of infiltrating macrophages and T lymphocytes, as well as fragmentation of collagen fibers. On day 21 of the experiment, a thin capsule was formed around ADM, there was a small number of infiltrating T lymphocytes and macrophages, the collagen fibers of the implant were intact. In the samples of the control group, there was pronounced inflammation with the presence of a significant number of lymphocytes and macrophages, as well as fragmentation and vascularization of the implant. On day 60 of the experiment, no inflammatory response was observed around ADM, biodegradation was minimal, and a dense fibrous capsule was formed around the fragment of the native porcine skin.

Conclusion. The experimental ADM has low immunogenicity and a low degree of biodegradation, which makes it possible to use it for further research to create efficient surgical material that is safe for use in clinical practice.

Keywords: regenerative medicine, skin, decellularization, morphological analysis, subcutaneous implantation, acellular dermal matrix

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

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Морфологический анализ местной тканевой реакции на подкожную имплантацию фрагментов ацеллюлярного дермального матрикса

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РЕЗЮМЕ

Ацеллюлярные дермальные матриксы (АДМ) набирают большую популярность в качестве хирургических материалов при операциях на органах малого таза, в ожоговой терапии и пластической хирургии. Проверка биосовместимости материалов является важным и необходимым этапом при разработке новых АДМ.

Цель исследования – провести сравнительный анализ результатов подкожной имплантации крысам АДМ и нативной дермы свиньи.

Материалы и методы. Для получения АДМ использовали детергентно-энзиматический метод децеллюляризации. Через 7, 14, 21, 60 сут после имплантации АДМ (экспериментальная группа) и нативной дермы свиньи (контрольная группа) животных выводили из эксперимента. Гистологические срезы окрашивали гематоксилином и эозином, трихромом по Массону, выполняли иммуногистохимическую реакцию с антителами к CD3 и CD68. Компьютерную морфометрию проводили с помощью программы ImageJ.

Результаты. На 7-е сут в экспериментальной группе отмечалось умеренное асептическое воспаление, в контрольной группе – выраженное асептическое воспаление с эозинофилами в инфильтрате. На 14-е сут в экспериментальной группе показано относительно низкое содержание макрофагов и Т-лимфоцитов с незначительным отеком, без биодеградации АДМ. В контрольной группе выявлено выраженное воспаление, инфильтрация большим количеством макрофагов и Т-лимфоцитов, а также фрагментация коллагеновых волокон. На 21-е сут вокруг АДМ сформировалась тонкая капсула, в инфильтрате малое количество Т-лимфоцитов и макрофагов, коллагеновые волокна имплантата были интактны. В образцах контрольной группы – выраженное воспаление с присутствием значительного количества лимфоцитов и макрофагов, фрагментация и васкуляризация имплантата. На 60-е сут вокруг АДМ воспалительной реакции не наблюдалось, биодеградация была минимальной, вокруг фрагмента нативной дермы свиньи сформировалась плотная фиброзная капсула.

Заключение. Разработанный АДМ обладает низкой иммуногенностью и малой степенью биодеградации. Это позволяет использовать данную конструкцию для дальнейших исследований по созданию полноценного хирургического материала, безопасного для применения в клинической практике.

Ключевые слова: регенеративная медицина, дерма, децеллюляризация, морфологический анализ, подкожная имплантация, ацеллюлярный дермальный матрикс

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

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INTRODUCTION

In recent years, researchers have widely used decellularized (acellular) materials to create tissue-engineered scaffolds and culture cells on them [1]. There are various methods of decellularization in regenerative medicine [2]. It is possible to create tissue-specific scaffolds that most accurately mimic the physical and chemical signals that are essential for cell adhesion, proliferation, migration, differentiation, and restoration of function [3].

Some of the most promising advances in surgical practice are acellular dermal matrices (ADMs) [4]. ADMs are gaining increasing popularity as surgical materials for operations on the pelvic organs, in burn therapy, and plastic surgery, including reconstructive mammoplasty [5]. In plastic surgery, they are used both in radical skin-sparing mastectomy and in secondary operations for breast deformities as materials supporting the endoprosthesis [6]. ADMs can be incorporated into the patient's connective tissues with further ingrowth of vessels and nerves and, therefore, function as these tissues [7]. The advantages of ADMs include the possibility to avoid the use of tissue expanders, reduction of postoperative pain, decrease in operation time, optimization of regeneration processes, and a better aesthetic result [7, 8]. A particular advantage of ADM is the presence of collagen as one of the main components of the dermis. Compared with scaffolds based on other biological or synthetic polymers, collagen scaffolds are optimal for cell growth and adhesion both *in vitro* and *in vivo*, have good biocompatibility and low immunogenicity, and make it possible to regulate biodegradation due to their ability to form complexes with biologically active substances. The latter stimulates proliferation of fibroblasts and formation of patient's own tissues [6].

There are several ADM-based surgical materials used in reconstructive surgery for soft tissue restoration – AlloDerm, Strattice, DermaMatrix, SurgiMend, Permacol, Veritas, and FlexHD. Some analogs cannot be used in Russian surgical practice due to legislation (AlloDerm, DermaMatrix, Dermalogen, AlloDerm, Cymetra), while materials from bovine collagen can cause a severe allergic reaction.

The most promising are ADMs based on porcine skin (Evolence, Strattice, Fibroquel, Permacol) that significantly reduce the risk of allergic complications in surgical practice [9]. The possibility of obtaining porcine skin from secondary raw materials increases the efficiency of the method for obtaining the material. Nevertheless, these ADMs are extremely expensive, which poses a challenge for modern regenerative medicine to create a more economical analog. The first stage in the creation of any ADM is assessment of its biocompatibility [10]. It should not damage cells or cause rejection, while its biomechanical properties should be comparable to those of native tissues. Therefore, to assess the characteristics of the matrix, it is necessary to compare early and long-term results of heterotopic xenotransplantation of the developed ADM and native porcine skin.

The aim of the study was to conduct a comparative morphological analysis of the results of subcutaneous implantation of the developed ADM and native porcine skin in rats.

MATERIALS AND METHODS

Samples of native skin of the Landrace pig with a thickness of 0.7 mm were taken after preliminary removal of the epithelial layer with a dermatome under sterile conditions and frozen at –80 °C. To obtain ADM, the skin was incubated for 6 hours (3 cycles, 2 hours each) at 37° C in a trypsin – Versene solution (Biolot, Russian Federation). Then, 2 treatment cycles were carried out with 1% Triton X-100 (SigmaAldrich, USA) and 4% sodium deoxycholate solution (SigmaAldrich, USA) combined with 0.002 M Na_2-EDTA for a total duration of 12 hours at room temperature in the incubator shaker (170 rpm). Then the samples were incubated in a solution of porcine pancreatic DNase (SigmaAldrich, USA; 2,000 U in 200 ml of phosphate-buffered saline (PBS)) at 37°

C for 4 hours. To ensure sterility of the samples, 1% solution of gentamicin and amphotericin B was added. The samples were washed with deionized water for 10 minutes between the cycles and at each change of solution. For the quantitative determination of residual DNA in the matrix, a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and a DNeasy Blood and Tissue Kit (Qiagen, Sweden) were used. To assess the quality of decellularization, the samples of decellularized skin were stained with hematoxylin – eosin (Histolab, Sweden) and DAPI fluorophore (4', 6-diamidino-2-phenylindole; Sigma Aldrich, USA).

Subcutaneous implantation of the samples was performed on male Wistar rats weighing 200–230 g and aged 6 months ($n = 32$). The samples (3 x 3 mm) were placed in a subcutaneous pocket on the withers. The animals were divided into the control and the experimental groups, each of which included 4 subgroups of 4 animals. The rats of the control group were implanted with samples of native porcine skin, and those of the experimental group – with ADM samples.

Biopsy material was taken for histologic and immunohistochemical analysis on day 7, 14, 21, and 60 after the subcutaneous implantation. The morphological analysis was performed on five independent biopsy fragments of the samples, for each of them nine sections were performed and analyzed. Tissue staining with hematoxylin – eosin and Masson's stain (BioVitrum, Russian Federation) was performed according to the standard protocol. Immunohistochemical study used heat-induced antigen retrieval, as well as polyclonal antibodies to T-lymphocyte receptor – CD3 (cat. Number ab11089, Abcam, UK) and to the macrosialin of monocytes and macrophages – CD68, (cat. Number ab955, Abcam, UK). All the samples before and after the implantation were examined using the Olympus CX 41 microscope (Olympus, Japan) at different magnifications. Computerized morphometry was performed using the ImageJ software (National Institution of Health, USA) and the IHC profiler. The diameter of the blood vessels and collagen fibers was assessed using the freehand selection tool. For quantitative analysis of the immunohistochemistry results, we used color segmentation with highlighting of the green channel, color binarization, and the particle analyzer tool.

Statistical processing of the data was performed using the MedCalc Statistical Software (Belgium). The Shapiro – Wilk test was used to check the nature of the distribution in the variational series. Since the distribution was different from normal, the results were presented as the median, as well as the first and the third quartiles Me [Q_1; Q_3]. The significance of differences was assessed using the Mann – Whitney U-test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The spectrophotometry data showed that the quantitative DNA content in the native porcine skin was 314.4 [300.7; 333.7] ng per 1 mg of tissue, and in ADM – 60.14 [55.34; 63.58] ng per 1 mg of tissue, which complied with the criteria for tissue decellularization quality [11]. The relatively low content of residual nucleic acids in ADM indicated low immunogenic potential of the matrix.

ADM had specific milky-white color; the histologic structure examination revealed no intact cells or nuclear fluorescence after DAPI staining (Fig. 1).

On day 7 after the implantation, pronounced sterile inflammation was registered in the animals of the experimental group. The immunohistochemical analysis and computerized morphometry detected 12.70 [12.19; 13.09] macrophages and 1.87 [1.56; 2.04] lymphocytes per 1 mm^2 of the section and an insignificant number of neutrophils in the cell infiltrate. Intact

Fig. 1. Stages of skin decellularization and histologic analysis of the resulting ADM: *a* – appearance of the native porcine skin, *b* – appearance of ADM, c – hematoxylin – eosin staining, no cell nuclei after decellularization; x20

collagen fibers of the implanted structure with thickness of 12.88 [11.87; 13.60] μm were clearly visible when stained with the Masson's trichrome stain. In the meantime, in the samples of the control group, pronounced sterile inflammation with an admixture of eosinophils in the inflammatory infiltrate and congestion of skin vessels and muscular arteries were identified. Infiltration of the implant with blood and inflammatory cells was noted – 9.03 [8.94; 9.16] macrophages and 1.91 [1.84; 1.99] lymphocytes per 1 mm^2 of the section (Fig. 2) were revealed after staining with the Masson's trichome. It was noted that the number of lymphocytes in the experimental group did not differ from that of the control group (p = 0.062), and the number of macrophages was significantly greater (p = 0.043) (Fig. 3).

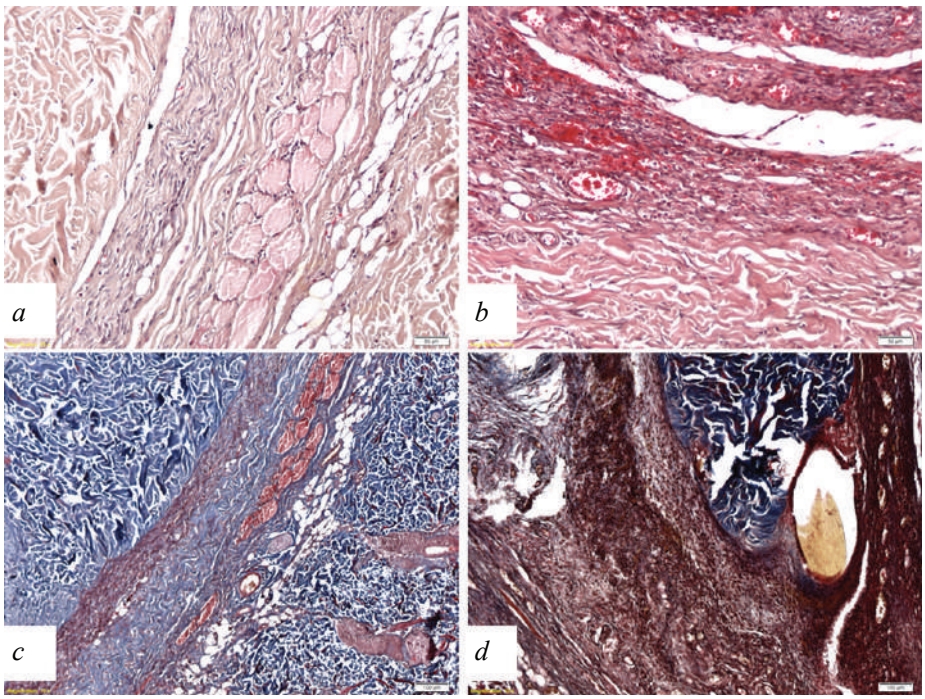

Fig. 2. Histologic evaluation of ADM and native porcine skin specimens after subcutaneous implantation in the experimental animals on day 7: *a* – ADM, hematoxylin – eosin, *b* – native porcine skin, hematoxylin – eosin, *c* – ADM, Masson's trichrome, *d* – native porcine skin, Masson's trichrome; x20

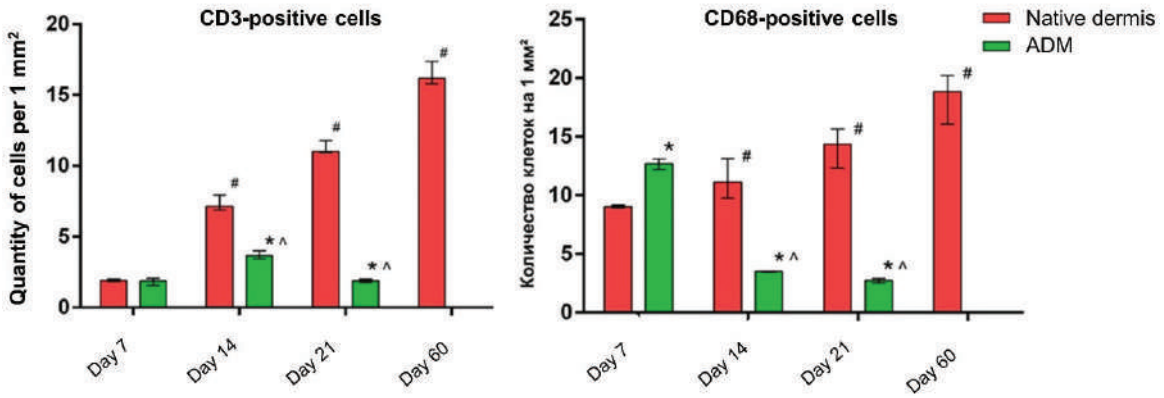

Fig. 3. Immunophenotyping of the inflammatory infiltrate at different periods of the experiment: *a* – changes in the content of T-lymphocytes (on the left), *b* – changes in the content of macrophages (on the right), *Me* [Q_1; Q_3]. * – the significance of the differences between the control and experimental groups, $p < 0.05$; # – the significance of the differences for the values in the control group for different periods, $p < 0.05$; ^ – the significance of the differences for the values in the experimental group for different periods, $p < 0.05$

On day 14, the formation of a thin connective tissue capsule around the implanted structure was noted in the experimental group. The thickness of the capsule was 18.17 [14.73; 20.32] μm, single macrophages (3.50 [3.44; 3.54] $CD68^+$ cells per 1 mm^2 of the section according to the computerized morphometry findings) were observed in the capsule walls. There was a thin layer of granulation tissue outside the capsule. Weakly pronounced vascular congestion was noted at the site of the implantation; the average diameter of the vessels was 30.97 [29.71; 31.82] μm. No evidence of degradation of ADM collagen fibers and no evidence of ADM infiltration by inflammatory cells were identified. We observed inflammation around the foreign body with predominance of a relatively large number of macrophages (11.13 [9.79; 13.11] $CD68^+$ cells according to the computerized morphometry data) in the samples of native porcine skin compared with the same parameter in the experimental group on day 14 ($p = 0.007$) and in the control group on day 7 ($p = 0.033$). Around the implant, a denser, thick-walled capsule with the average thickness of 165.2 [152.90; 188.80] μm, compared with the experimental group ($p = 0.003$), was formed.

The collagen fibers of the implant were fragmented, edematous, infiltrated with blood with an admixture of neutrophils and macrophages, and characterized by a pronounced oxyphilic reaction of the medium. A large number of thin congested vessels were found in immediate proximity to the capsule (the average diameter was 20.81 [19.41; 21.61] μm) (Fig. 4). There was a greater number of CD3-positive cells in both groups relative to the previous period of sample explantation – 3.66 [3.42; 4.01] cells per 1 mm^2 of the section for the experimental group ($p = 0.039$) and 7.14 [6.87; 7.93] cells per 1 mm^2 of the section for the control group ($p = 0.041$).

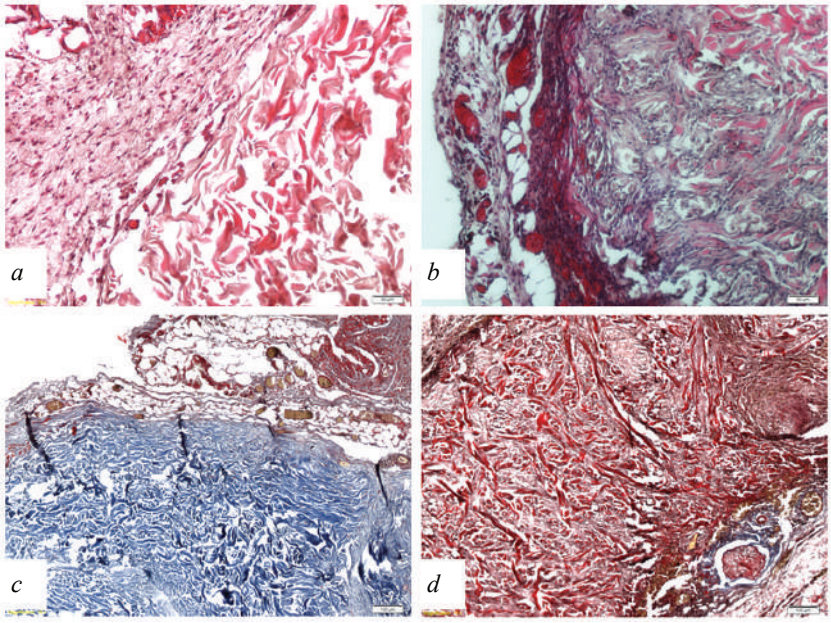

Fig. 4. Histologic evaluation of ADM and native porcine skin specimens after subcutaneous implantation in the experimental animals on day 14: *a* – ADM, hematoxylin – eosin, *b* – native porcine skin, hematoxylin – eosin, *c* – ADM, Masson's trichrome, *d* – native porcine skin, Masson's trichrome; x20

On day 21 after the ADM implantation, the thickness of the capsule around the implant was 10.02 [9.95; 10.10] μm, and a small number of inflammatory cells was observed – 2.73 [2.54; 2.89] $CD68^+$ macrophages and 1.90 [1.76; 1.99] $CD3^+$ lymphocytes per 1 mm^2 of the section, which was significantly lower than in the control group ($p = 0.012$ and $p = 0.009$, respectively). The average capillary diameter was 26.61 [19.48; 31.52] μm; no congested vessels were observed. The collagen fibers of the encapsulated ADM were completely preserved and had the diameter of 11.67 [10.02; 14.50] μm. There was no accumulation of leukocytes in the ADM or its infiltration with blood. At the same time, the formation of a dense connective tissue capsule with the thickness of 66.53 [61.24; 7.59] μm was observed in the animals of the control group. Numerous small clusters of T-lymphocytes, CD68-positive macrophages (the content was 14.37 [12.33; 15.65] cells per 1 mm^2 of the section), and foreign body cells were detected. Moreover, along with pronounced encapsulation, the presence of congested vessels with the diameter of 23.80 [19.24;

27.76] μm was noted inside the sample, which indicated active sample biodegradation. The collagen fibers were swollen and partially destroyed – their thickness ranged from 5 to 37 μm. Macrophages and T-lymphocytes were present in the thickness of the implant, which also confirmed immune rejection.

On day 60 after the subcutaneous implantation, the ADM was surrounded by the connective tissue capsule (its thickness was 13.83 [12.03; 15.54] μm), and inflammation was completely absent. Immunohistochemistry did not show the presence of macrophages and T-lymphocytes at the implantation site. Atrophy of surrounding tissues was absent; focal reactive lipomatosis was noted. The collagen fibers of the implant with the diameter of 15.06 [12.45; 15.99] μm were completely preserved, biodegradation was minimal. In the control group, significant implant degradation due to its lysis by macrophages and persistent perifocal inflammation were observed. A quantitative assessment showed the presence of 15.79 [14.50; 17.67] T-lymphocytes and 18.86 [16.09; 20.22] macrophages per 1 mm^2 of the section, which was significantly higher than the previous control value ($p = 0.008$ and $p = 0.048$, respectively). A multilayer capsule with the thickness of 107.20 [91.32; 117.50] μm formed around the fragment of the native porcine skin, the vessels surrounding it were congested and had the diameter of 28.50 [26.32; 31.45] μm (Fig. 5).

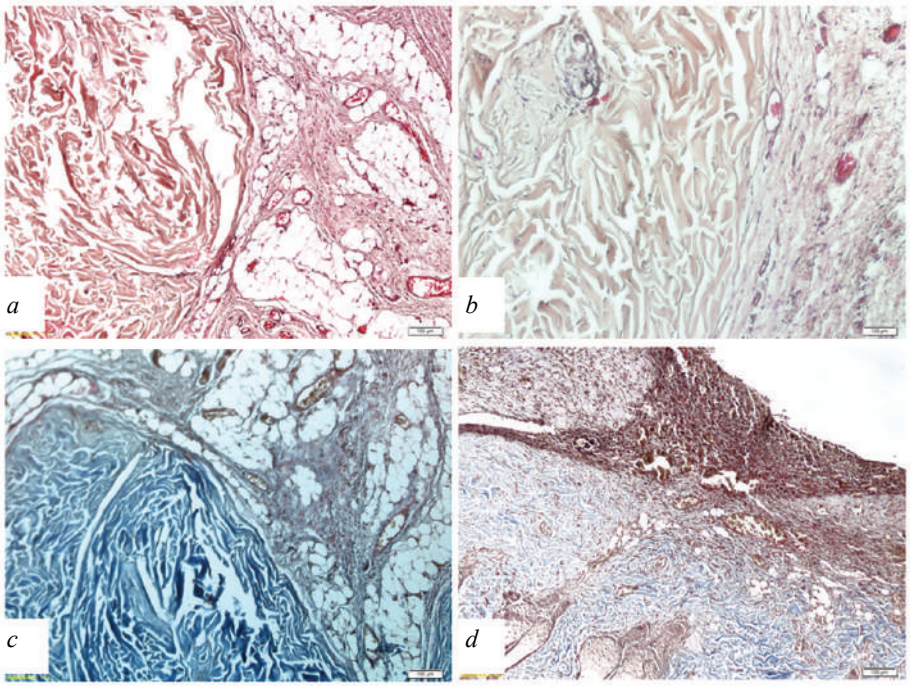

Fig. 5. Histologic evaluation of ADM and native porcine dermis specimens after subcutaneous implantation in the experimental animals on day 60: *a* – ADM, hematoxylin – eosin, *b* – native porcine skin, hematoxylin – eosin, *c* – ADM, Masson's trichrome, *d* – native porcine skin, Masson's trichrome; x20

Therefore, the results of immunophenotyping of the inflammatory infiltrate around and directly inside the implant at different periods of the experiment suggested that the ADM had minimal immunogenicity compared with the native porcine skin. A relatively weak inflammatory response and a thin connective tissue capsule around the implanted fragment, noted at later stages of the experiment, confirmed low antigenic properties of the ADM. Collagen fibers, which are the main component of the extracellular matrix that determine its mechanical properties, remained practically intact throughout the entire experiment. In the meantime, the implanted native porcine skin underwent significant biodegradation due to a pronounced inflammatory response, which was confirmed by the fragmentation of collagen fibers, the presence of congested capillaries, and a dense connective tissue capsule surrounding the implanted fragment.

CONCLUSION

The study showed that the developed ADM was characterized by low immunogenicity and a low degree of biodegradation. It allows to test the protocol of its development and use this construct as a starting point for further research on the biological and biomechanical properties of ADM to create valuable surgical material that is safe for application in clinical practice.

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Authors contribution

Melkonyan K.I., Rusinova T.V. – conception and design, substantiation of the manuscript and critical revision of the manuscript for important intellectual content. Verevkin A.A., Sotnichenko A.S. – carrying out of the experiment, analysis and interpretation of the data, drafting of the manuscript. Kozmay Ya.A., Asyakina A.A., Kartashevskaya M.I. – review of literature on the topic of the article, analysis and interpretation of the data. Gurevich K.G., Bykov I.M. – final approval of the manuscript for publication.

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Application of screening techniques for early diagnosis of a risk of bipolar disorder in adolescents

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ABSTRACT

Aim. To assess the possibility of combined application of screening methods for early detection of risks of bipolar disorder in adolescents.

Materials and methods. The study included 139 adolescents aged 13–16 years. A clinical psychopathology assessment as well as screening methods were used. The screening methods included the Bipolar Spectrum Diagnostic Scale ((BSDS), R. Pies, 2005) and the Mood Disorder Questionnaire ((MDQ), R.M. Hirschfeld, 2000).

Results. The clinical psychopathology assessment was performed in accordance with criteria of ICD-10, Class V. No mental and behavioral disorders (F00-F99), including affective pathology, were identified. Following the MDQ screening, the risk of bipolar disorder was revealed in 63 individuals (45.3%; 95 % confidence interval (CI): (36.8–53.9). When the BSDS method was used, the risk of bipolar disorder was revealed in 16.2% of cases (CI: (11.9–28.3)). The combined use of the screening scales (MDQ and BSDS) confirmed their consistency in detecting values both not exceeding (48.7% of the cases) and exceeding the threshold rates (17.1% of the cases).

Conclusion. Early diagnosis of a risk of bipolar disorder in adolescents, along with a clinical psychopathology assessment, may include application of screening scales. Combined use of several screening methods is justified by polymorphism of initial hypomanic and depressive states, as well as by difficulties in subjective assessment of symptoms of bipolar disorder in adolescents.

Keywords: adolescents, bipolar disorder, hypomania, screening methods

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Conformity with the principles of ethics. Parents of adolescents signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Smolensk State Medical University (Protocol No. 3 of 02.12.2018).

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Применение скрининговых методик для ранней диагностики риска биполярного аффективного расстройства у подростков

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РЕЗЮМЕ

Цель. Изучение возможности комплексного применения скрининговых методик в ранней диагностике риска биполярного расстройства (БР) у подростков.

Материалы и методы. В исследовании приняли участие 139 подростков в возрасте 13–16 лет. Были использованы клинико-психопатологический метод и скрининговые методы исследования: диагностическая шкала расстройств биполярного спектра (Bipolar Spectrum Diagnostic Scale, *BSDS*, R. Pies, 2005); вопросник расстройства настроения (Mood Disorder Questionnaire MDQ, R.M. Hirschfeld, 2000).

Результаты. При клинико-психопатологическом исследовании подростков в соответствии с критериями МКБ-10 (класс V: психические расстройства и расстройства поведения (F00-F99)) психические расстройства, в том числе аффективная патология, не выявлены. По результатам скрининговой методики MDQ показан риск БР у 63 респондентов (45,3%; 95-й доверительный интервал (ДИ): 36,8–53,9). С помощью методики BSDS риск БР отмечен у 16,2% респондентов (ДИ: 11,9–28,3). Совместное использование скрининговых шкал (MDQ и BSDS) продемонстрировало согласованность их работы по выявлению значений, как не превышающих пороговые (48,7% случаев), так и превышающих пороговые показатели (17,1% случаев).

Заключение. Ранняя диагностика риска БР у подростков наряду с клинико-психопатологическим методом может включать использование скрининговых шкал. Комплексное использование нескольких скрининговых методов обосновано проблемой полиморфизма начальных гипоманиакальных и депрессивных состояний, а также трудностями субъективной оценки симптомов биполярного аффективного расстройства подростками.

Ключевые слова: подростки, биполярное аффективное расстройство, гипомания, скрининговые методы

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. Информированное согласие подписано родителями подростков по соответствующей форме. Исследование одобрено этическим комитетом СГМУ (протокол № 3 от 02.12.2018).

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INTRODUCTION

Bipolar disorder (BD) is a chronic, almost lifelong disease involving not only episodes of mood disorders (subdepression, depression, hypomania, mania, and mixed episodes), but also a wide range of comorbid disorders, decreased neurocognitive functions, and a significant decline in social functioning [1].

BD is one of the leading causes of disability in the population and is associated with high rates of premature mortality, both from suicide [2] and concomitant diseases [3, 4]. Among patients with BD, especially those with mixed episodes and psychotic disorders, the risk of suicidal behavior is one of the highest among all mental disorders, especially in adolescence [5].

BD is characterized by high prevalence in the general population (from 0.6 to 5.84%), and with account of subsyndromal manifestations, it reaches 12% [6]. The issue of studying BD manifesting in adolescence seems relevant in the light of the increase in the proportion of this pathology in puberty [7, 8].

An important aspect of this issue is a difficulty in diagnosis, largely associated with the atypical clinical presentation of subdepressive, depressive, hypomanic, manic, and mixed episodes debuting in adolescence, its polymorphism, and syndrome incompleteness due to the characteristics of the developing adolescent psyche [9]. This leads to the fact that a significant part (up to 70–80%) of adolescents suffering from BD do not receive timely adequate treatment or do not come to the attention of a psychotherapist or psychiatrist at all [10].

Depression is a frequent clinical manifestation of most mental illnesses at puberty, especially at their initial stage, which is probably due to the prevalence of the emotional and ideological response at this stage of ontogenesis [11, 12].

Hypomanic states often precede BD in adolescence [13]. A significant problem is differentiation between hyperthymic temperament, as an extreme variant of the norm, and hypomanic states in BD. In addition, adolescent behavioral features that determine developmental crisis during puberty (reaction of emancipation, opposition, grouping with peers, etc.) [12] are often associated with the onset of substance abuse, which may not exclude early symptoms of hypomania [13, 14].

Hypomania presents significant problems in the diagnosis of bipolar II disorder. Hypomanic states are difficult to recognize both by patients themselves and their relatives; most patients do not consider such states painful and, therefore, do not seek medical care [15]. This is especially true for adolescents, who may enjoy a state of heightened mood and elation. They can exacerbate this state by taking psychoactive substances followed by risky behavior [16].

Irritability, as one of the diagnostic criteria for mood disorders (hypomania, mania, subdepression, depression), is also quite common in the prepubertal and pubertal developmental periods. Irritability is often accompanied by various forms of aggressive behavior in adolescents and requires thorough differential diagnosis. In the study by F. Benazzi, H. Akiskal (2004), irritability was more often detected in bipolar II disorder. BD with irritability had an earlier onset, high comorbidity rates, as well as aggravated family history for BD [17].

Over the decades, international diagnostic systems have changed approaches to the diagnosis of hypomania, as one of the criteria for bipolar II disorder. This concerned both the duration of the hypomanic episode and the number of symptoms it should correspond to. The ICD-11 project includes changes concerning the diagnosis of a hypomanic episode [18–20].

J. Angst et al. (2020) conducted a comparative analysis of diagnostic criteria for BD using DSM-V (APA, 2013) and the ICD-11 project. They noted that, unlike DSM-IV-TR and ICD-10, in DSM-V and the ICD-11 project, among the main criteria for diagnosing a hypomanic episode, in addition to mood changes (euphoria, irritability), the emphasis is placed on increasing activity, a surge of strength, and a subjective feeling of vitality, which, according to the authors, is essential to describe a more complete and accurate clinical presentation of the disorder. In the DSM-V, the number of additional symptoms for the diagnosis of hypomania is limited to three or more for increased mood and to four or more for irritability. In the ICD-11 project, “a few” additional symptoms are necessary to make a diagnosis. The differences also concern the duration of the hypomanic episode: in DSM-V, symptoms must be present for at least 4 days, while there are no such restrictions in the ICD-11 project, and a period of “several days” is mentioned.

J. Angst et al. (2020) note that the diagnostic criteria for a hypomanic episode in the ICD-11 project enable to detect it twice more often than in DSM-V. The issues requiring further study are social and psychological consequences of hypomania which are not always subjectively undesirable for the patient (increased efficiency, acceleration of associative processes, reduced duration of night sleep, increased creative abilities). The authors point out that for a more accurate diagnosis, it is necessary to take into account episodes of seeking outpatient medical care, which may be associated to a greater extent with the negative consequences of a hypomanic episode and will maximize objectification of the patient’s mental state [21].

Researchers indicate that the average age at the onset of BD varies from 20 to 30 years. Some authors note two peaks of BD debut: 15–24 years and 45–54 years. There are also indications of an earlier (up to 12 years) onset of the disease, associated, among other things, with the impact of a traumatic event [22]. Certain BD symptoms in the form of cyclothymic mood swings can be detected in adolescence and are characterized by a risk of progression to BD [23, 24].

Researchers are also discussing the diagnosis of prepubertal bipolar disorder (PPBD) [25]. However, clinical manifestations of the main BD symptoms in childhood differ significantly from those in adults, and in some cases are not similar. Mixed episodes that are difficult to diagnose and are common in adults, can also occur in childhood and adolescence, which further complicates early diagnosis of affective pathology in these age periods [26].

At the onset of BD in childhood, the disease is characterized not only by a more unfavorable course compared with the debut in adults (more episodes, substance use and disability), but also by a longer delay in the initiation of treatment. J.S. Kroon et al. (2013) found that the first episode of BD that occurred at the age of 15–24 years subsequently contributed to a more severe course of the disease in patients aged 45–54 years [27].

BD in adolescents is often complicated by comorbid mental and somatic disorders that significantly increase the risk of suicidal behavior. This primarily concerns substance abuse. According to M.H. Swahn et al. (2007), early initiation of alcohol consumption (especially at the prepubertal age) statistically significantly increases the risk of suicidal thoughts and can provoke suicidal attempts in both boys and girls [28].

Researchers attribute great importance to identifying prodromal symptoms that precede the onset of the disease. According to the study by G.A. Fava et al. (2007), the majority of patients had such symptoms as difficulty with falling asleep, irritability, and anxiety before the onset of clinically defined syndromes [29]. A.R. Van Meter et al. (2016) noted that more than half of the respondents had a symptom of a significant increase in energy before the onset of a manic episode [30]. There is evidence that behavioral disorders, aggressiveness, and impulsivity in adolescence also precede BD [31].

Along with the main clinical and psychopathological method, screening tools are widely used for the diagnosis of BD, especially for timely detection of hypomanic episodes. These include the Mood Disorder Questionnaire (MDQ) developed in 2000 by a team led by R.M.A. Hirschfeld [32], the Bipolar Spectrum Diagnostic Scale (BSDS) developed by R. Pies and improved by a group of researchers led by S.N. Ghaemi in 2005 [33], and the 32-item Hypomania Checklist (HCL-32) [34] proposed by J. Angst et al. in 2005 and validated in Russia by S.N. Mosolov et al. [35]. These screening methods are used in both clinical and non-clinical populations.

Thus, studies on hypomania in a non-clinical adolescent population using HCL-32 showed the relationship of hypomania with sleep disorders and personality traits, which confirms the validity of using this screening method as a tool for identifying adolescents at risk of developing BD [36]. A. Päären et al. (2012), studying adolescents (n = 2,300) with a positive screening result for hypomania, upon re-examination after 15 years, revealed hypomanic episodes in 3–6% of cases [37].

According to many researchers, the use of screening techniques in non-clinical populations to identify the risk of BD causes many difficulties, since the sensitivity and specificity parameters vary widely. A subjective assessment of the emotional state (hypomania) in adolescents is also complicated. In addition, the debut of BD is characterized by a high degree of polymorphism, which poses additional challenges for the diagnosis of the disease at early stages [38].

MATERIALS AND METHODS

The aim of the study was to investigate diagnostic capabilities of combined use of the Mood Disorder Questionnaire (MDQ) and the Bipolar Spectrum Diagnostic Scale (BSDS) to identify the risk of BD in a non-clinical adolescent population.

The study involved 139 adolescents studying at a specialized school in the Smolensk region within the programs of the Federal State Educational Standard of Secondary (Complete) General Education (57 (41.1%) boys and 82 (58.9 %) girls). The average age was 14.61 ± 0.09 years (min = 13; max = 16). The study participants were comparable in age and social and educational status. No significant differences were found by gender ($p > 0.05$).

The informed consent was signed by the parents of the adolescents according to the standard form. The study was conducted in groups of 15–20 people. A preliminary permission had been received to ask clarifying questions from a psychiatrist present during the study. In most cases, adolescents did not have any difficulties with filling out the questionnaire. On average, it took them 30 minutes to fill out the printed copy of the screening questionnaires.

The clinical and psychopathological method and screening methods were used in the work: the Bipolar Spectrum Diagnostic Scale (BSDS; R. Pies, 2005) [33]; the Mood Disorder Questionnaire (MDQ; R.M.A. Hirschfeld, 2000) [32].

Statistical processing of the data included methods of descriptive statistics. The 95% confidence interval (CI) was calculated using the Wald's equation; for small values, it was calculated using the Wald's equation modified according to the method of Agresti – Coull. The significance of the differences between the studied features was assessed using the Pearson's chi-squared test (χ^2) and Fisher transformation. Statistical significance was recognized with a probability of >95% ($p < 0.05$). The relationship between the features was assessed by Spearman's rank correlation coefficient. Statistical analysis of the results was performed in Microsoft Excel 16 using the Data Analysis and AtteStat add-ons and the R statistical package.

RESULTS

During the clinical and psychopathological assessment of the adolescents in accordance with the criteria of ICD-10, class V, mental and behavioral disorders (F00–F99) and mental disorders, including affective pathology, were not identified.

The next stage of the research was to study the risk of developing BD using the Mood Disorder Questionnaire (MDQ). 139 adolescents took part in it. The MDQ consists of 3 sections: the first includes 13 questions reflecting symptoms of mania (hypomania), the second registers the simultaneous presence of one or more symptoms, and the third registers the degree of habitual activity interference due to the presence of symptoms. The average value of positive answers among the respondents in this sample was 7.14 ± 0.26 points (*min* = 1; *max* = 15). These values do not exceed the screening threshold of 7 points [20]. The results of the study are presented in Table 1.

Table 1

The results of the distribution of positive responses according to the MDQ scale, *n* = 139

The distribution of positive answers according to the MDQ scale, points	The number of the respondents who gave a positive answer, *n* (%)	95% CI
15	1 (0.72)	0.13–3.96
14	2 (1.44)	0.4–5.1
13	3 (2.16)	0.74–6.16
12	3 (2.16)	0.74–6.16
11	12 (8.63)	3.96–13.30
10	12 (8.63)	3.96–13.30
9	14 (10.07)	5.07–15.08
8	16 (11.51)	6.21–16.82
7	16 (11.51)	6.21–16.82
6	16 (11.51)	6.21–16.82
5	16 (11.51)	6.21–16.82
4	9 (6.47)	2.38–10.57
3	10 (7.19)	2.90–11.49
2	6 (4.32)	0.94–7.70
1	3 (2.16)	0.74–6.16

Note: 95% CI with respect to the relative rate of a positive response, $p < 0.05$ (here and in Table 2).

To identify the proportion of respondents with an increased level of screening values, grouping was carried out according to the screening threshold. The respondents were divided into two groups. The first group included 76 respondents, which is 54.7% (CI: 46.1–63.1), who scored 7 or less points (not exceeding the screening threshold); the second group included respondents in the amount of 63 individuals, which is 45.3% (CI: 36.8–53.9), who scored more than 7 points (exceeding the screening threshold).

No significant differences were found between the groups φ *emp = 1.329 ($p > 0.05$), i.e. the proportion of the respondents with screening values not exceeding the screening threshold does not significantly differ from the proportion of the respondents exceeding the screening threshold; the respondents of both groups are seen in the studied social environment with equal likelihood.

The next stage of the study was to investigate the possibilities of using the Bipolar Spectrum Diagnostic Scale (BSDS) screening technique to identify the risk of developing BD. From 139 individuals, the study involved 99 adolescents aged 13–16 years.

BSDS is a technique that, along with the manifestations of depression, takes into account the symptoms of hypomania (mania). The respondent's agreement with any of the proposed statements is equal to 1 point. The total BSDS score can be in the range

of 0–25. The probability of BD is estimated according to the sum of points: 20 points or more – bipolar spectrum disorder is very likely, 13–19 points – a moderate probability of bipolar spectrum disorders, 7–11 points – low probability of bipolar spectrum disorders, less than 7 points – BD is unlikely. The screening threshold is 13 points.

The average score for positive responses among the respondents in this population was 8.93 ± 0.39 (*min* = 1; *max* = 20), which is below the screening threshold (13 points). The results of the study are presented in Table 2.

Table 2

The results of the distribution of the total score for positive responses according to the BSDS, *n* = 99

The total score for positive responses according to the MDQ scale, points	The number of respondents who gave a positive answer, *n* (%)	95% CI
20 and more	1 (1.01)	0.18–5.5
13–19	28 (28.28)	20.35–37.83
7–11	50 (50.51)	40.83–60.15
less than 7	20 (20.20)	13.47–29.15

The table shows the prevalence of low (50.51%) and moderate (28.28%) trends toward BD in the respondents. To identify the proportion of the respondents with an increased level of screening values (13 or more points), grouping was carried out according to the screening threshold. The respondents were divided into two groups. The first group included 83 respondents, which is 83.8%, CI: (75.1–90.5), who scored less than 13 points (not exceeding the screening threshold); the second group consisted of 16 individuals, which is 16.2%, CI: (11.9–28.3), who scored 13 or more points (exceeding the screening threshold).

When comparing the two groups, significant differences were revealed φ *emp = 10.501 ($p < 0.05$), i.e. the proportion of respondents with values not exceeding the screening threshold significantly differs from the proportion of respondents exceeding the screening threshold. This sample is dominated by respondents with values not exceeding the screening threshold (less than 13 points), respectively, who do not have a risk of developing BD.

The next stage of the research was to study the simultaneous (combined) use of the MDQ and BSDS screening techniques; a comparative analysis was carried out. As a result, the MDQ scale revealed a tendency toward BD in a non-clinical population of adolescents to a greater extent than the BSDS; φ *emp = 6.36 ($p < 0.05$) (Fig. 1). The average value according to the MDQ scale approached the screening threshold value of 7 points (7.14 ± 0.26, min = 1; max = 15), and the average value according to the BSDS (8.93 ± 0.39, min = 1; max = 20) was 13 points lower than the screening threshold. The results obtained may indicate difficulties experienced by adolescents in assessing the emotional state, which justifies a comprehensive approach to the diagnosis of a tendency toward affective pathology.

Further, we performed a correlation analysis of the survey results on the BSDS and MDQ scales. A noticeable direct, positive relationship was revealed ($r = 0.55$; $p < 0.05$), which indicates the possibility of combined use of these screening scales (Fig. 2).

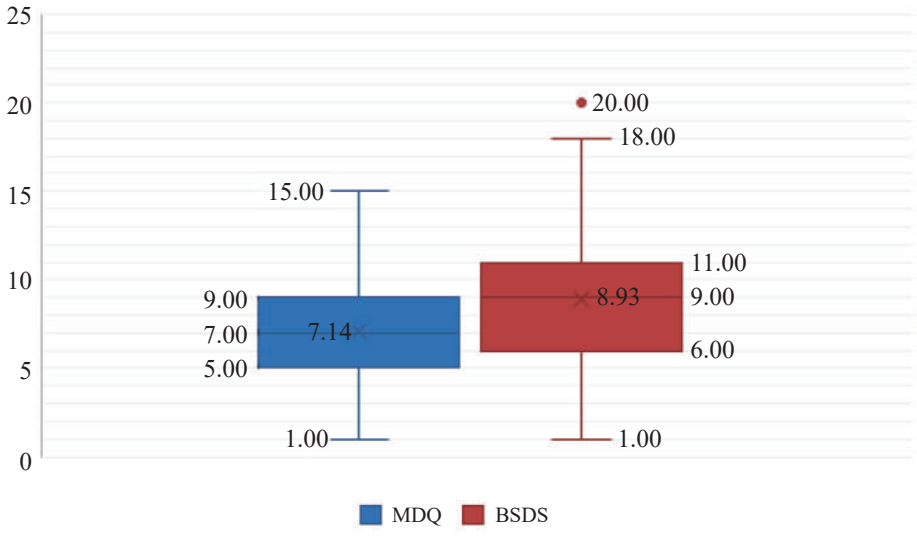

Fig. 1. Distribution of average values on the BSDS and MDQ scales

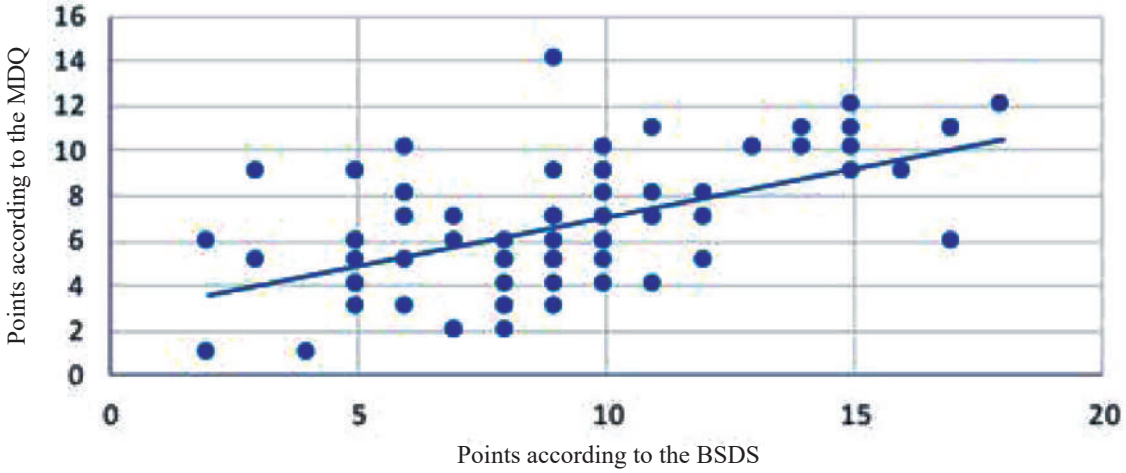

Fig. 2. Study of the correlation between the MDQ and BSDS

To assess the possibility of combined use of the two scales, the Pearson's test was used. When analyzing the statistical relationship between the parameters of the MDQ and BSDS screening methods (above / below the screening value for both scales), $\chi 2$ was 12.6, which indicates a high degree of its statistical significance (p = 0.0004). Combined use of the two techniques in the groups exceeding the screening threshold was revealed in 17.1% of cases. In the groups of respondents where the screening values on both scales did not exceed the threshold values, the combined use of the methods was detected in 48.7% of cases.

The data obtained may indicate the effectiveness of comprehensive research in non-clinical samples. In this study, the combined use of the two screening scales (MDQ and BSDS) revealed a risk of developing affective disorders in 17% of the respondents. At the same time, with the use of each of these techniques separately, contradictory results were obtained: according to the MDQ data, a tendency toward affective pathology was revealed in 45.3% of the respondents, while according to the BSDS, only 16.2% of the respondents in the non-clinical sample were found to exceed the screening threshold.

The findings obtained are consistent with the results of studies on possibilities of applying screening scales in the general population. Scholars discuss a significant variation in the sensitivity and specificity of screening methods in non-clinical samples. R.M. Hirschfeld et al. (2003), when studying validity of the MDQ scale for application in the general population, found that the sensitivity of the method was 28.1% and the specificity was 97.2%, which significantly limits its use [39].

The study of possibilities of the BSDS in outpatient psychiatric practice shows greater sensitivity with regard to exclusion of the diagnosis of BD. At the same time, some experts consider the BSDS a useful screening tool for detecting subclinical manifestations of hypomania [40]. In order to optimize screening for BD, according to many researchers, the combined use of several diagnostic questionnaires may be recommended. An increase in the efficiency of BD screening was shown with the combined use of the MDQ and the HCL-32, the BSDS and the HCL-32, as well as the BSDS and the MDQ [41, 42].

Therefore, this analysis has shown that the combined use of two screening scales and clinical and psychopathological assessment can contribute to more reliable results in detecting mood disorders in non-clinical populations of adolescents and minimize the problem of over- and underdiagnosis of subdepression and hypomanic states.

CONCLUSION

In the clinical and psychopathological assessment of adolescents in accordance with the criteria of ICD-10, class V (mental disorders and behavioral disorders (F00–F99)), mental disorders, including affective pathology, were not detected. Based on the results obtained, it may be assumed that in this study, the MDQ and BSDS screening methods can work in unison, to detect respondents with values both not exceeding and exceeding the screening threshold. In particular, consistency of measurements of the methods in iden-

tifying values that do not exceed the screening threshold is confirmed in 48.7% of cases, which indicates that there is no risk of BD in the studied population of healthy adolescents. In 17.1% of cases, the consistency of measurements of the two methods in identifying the excess of the screening threshold corresponds to the risk of developing BD in this population of respondents.

Identifying the risk of BD in adolescents is very complicated due to significant polymorphism of both prodromal and initial manifestations of BD. Adolescence, characterized by complex neuroendocrine changes in the body, also causes certain alterations in the initial manifestations of BD. Psychological features typical of adolescence (reactions of emancipation, opposition; active and passive protest; aggravation of characterologic traits, etc.) can act both as psychological phenomena and psychopathological symptoms and require thorough differential diagnosis.

Early diagnosis of the risk of BD in adolescents, along with the clinical and psychopathological assessment, may include the use of screening scales. The combined use of several screening methods is justified by polymorphism of initial hypomanic and depressive states, as well as the by difficulties in subjective assessment of BD symptoms by adolescents. Further research on possibilities of early diagnosis of the risk of BD requires a comprehensive approach with use of the basic clinical and psychopathological assessment and additional psychometric scales, a thorough assessment of anamnestic data, and subsequent follow-up.

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Serum concentrations of interleukin-6 and tumor necrosis factor alpha in patients with spondyloarthritis: a relationship between systemic inflammation and anemia

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ABSTRACT

Aim. To assess the relationship between the activity of systemic inflammation and the hemoglobin level in patients with spondyloarthritis (SpA).

Materials and methods. We examined 92 patients with SpA aged 42.9 ± 11.6 years (SpA duration – 14.8 ± 9.6 years, 55 (60%) men). We calculated the BASDAI and ASDAS-CRP scores, performed complete blood count, evaluated erythrocyte sedimentation rate (ESR), ferrokinetic parameters, C-reactive protein (CRP) level, and serum concentrations of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6).

Results. Anemia was found in 52 (57%) patients: 13 (25%) patients were diagnosed with anemia of chronic disease (ACD), 39 (75%) individuals had a combination of ACD and iron deficiency anemia. A significant increase in CRP (17.8 vs. 9.0 mg / l, respectively; $p = 0.001$) and ESR (23 vs. 10 mm / h, $p < 0.001$), a tendency toward an increase in IL-6 levels (5.4 vs. 4.1 pg / ml, $p = 0.051$), and no difference in TNF-α levels (3.4 vs. 3.0 pg / ml, $p = 0.245$) were revealed in patients with anemia compared with patients without the disease. The hemoglobin concentration was negatively correlated with the CRP level ($r = –0.327$, $p = 0.001$) and ESR ($r = –0.527$, $p < 0.001$). IL-6 was positively correlated with the levels of TNF-a, CRP, and ESR ($r = 0.431$, $r = 0.361$, $r = 0.369$; all $p < 0.001$). With the IL-6 concentration >10 pg / ml, the odds for anemia were 5.3 times higher (95% confidence interval: 1.4–19.9, $p = 0.009$).

Conclusion. The relationship between the activity of systemic inflammation and anemia in patients with SpA was confirmed. Taking into account the pathogenesis of ACD, the aim of antianemic treatment is to achieve remission or minimal activity of SpA. Additional studies are required to determine the effect of backbone anti-inflammatory therapy on the development and course of anemia in patients with SpA.

Keywords: anemia, hemoglobin, inflammation, interleukin-6, tumor necrosis factor α, spondyloarthritis

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Сывороточная концентрация интерлейкина-6 и фактора некроза опухоли α у пациентов со спондилоартритами: связь между системным воспалением и анемией

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РЕЗЮМЕ

Цель – оценить взаимосвязи между лабораторной активностью системного воспаления и уровнем гемоглобина у пациентов со спондилоартритами (СпА).

Материалы и методы. Обследованы 92 пациента со СпА (возраст – 42,9 ± 11,6 года, длительность СпА – 14,8 ± 9,6 лет, 55 (60%) мужчин). Рассчитаны индексы BASDAI, ASDAS-CRP, исследованы клинический анализ крови, скорость оседания эритроцитов (СОЭ), параметры феррокинетики, уровень С-реактивного белка (СРБ), сывороточная концентрация фактора некроза опухоли α (ФНО-α) и интерлейкина-6 (ИЛ-6).

Результаты. У 52 (57%) пациентов выявлена анемия: у 13 (25%) диагностирована анемия хронического воспаления (АХВ), у 39 (75%) – комбинация АХВ и железодефицитной анемии. У пациентов с анемией по сравнению с больными без анемического синдрома отмечено статистически значимое увеличение уровня СРБ (17,8 и 9,0 мг/л соответственно, $p = 0,001$) и СОЭ (23 и 10 мм/ч соответственно, $p < 0,001$), установлена тенденция к повышению уровня ИЛ-6 (5,4 и 4,1 пг/мл соответственно, $p = 0,051$), концентрация ФНО-α статистически значимо не различалась (3,4 и 3,0 пг/мл, $p = 0,245$). Установлена обратная взаимосвязь между уровнем гемоглобина и уровнем СРБ ($r = –0,327$; $p = 0,001$), СОЭ ($r = –0,527$; $p < 0,001$). Концентрация ИЛ-6 статистически значимо взаимосвязана с уровнем ФНО-α, СРБ и СОЭ ($r = 0,431$; $r = 0,361$; $r = 0,369$; $p < 0,001$ для всех). При концентрации ИЛ-6 >10 пг/мл шансы развития анемии у пациентов со СпА увеличивались в 5,3 раза (95%-й доверительный интервал 1,4–19,9; $p = 0,009$).

Заключение. В ходе исследования подтверждена взаимосвязь между лабораторной активностью системного воспаления и анемией у больных СпА. Учитывая патогенез АХВ, основой антианемической терапии является достижение ремиссии, а при невозможности – минимальной активности СпА. Требуется проведение дополнительных исследований для определения влияния базисной противовоспалительной терапии на развитие и течение анемии у пациентов со СпА.

Ключевые слова: анемия, гемоглобин, воспаление, интерлейкин-6, фактор некроза опухоли α, спондилоартрит

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

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

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Проведение исследования одобрено этическим комитетом Саратовского ГМУ им. В.И. Разумовского (протокол № 3 от 07.11.2017).

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INTRODUCTION

Anemia of chronic disease (ACD) is the second most prevalent type of anemia after iron deficiency anemia (IDA) and is characterized by high prevalence among different groups of patients [1–3]. Anemia is a common comorbid pathology in patients with rheumatic diseases, which is associated with the pathogenesis of autoimmune diseases [4]. Overproduction of interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) plays a key role in the pathogenesis of ACD and leads to impaired iron homeostasis and erythropoiesis [4, 5].

Significant effects of IL-6 include stimulation of hepcidin production in the liver. Increased levels of circulating hepcidin result in suppression of nutritional iron absorption in the duodenum and impairment of endogenous iron recirculation due to its sequestration in cells of the macrophage – monocyte system [4, 5]. TNF-α directly inhibits intestinal iron absorption by the hepcidin-independent mechanism, reduces the erythrocyte life span, promotes erythrophagocytosis, suppresses proliferation of erythroid progenitor cells via implementation of proapoptoptic effects, and inhibits erythropoietin production [4, 5]. Following the combined effect of hepcidin and cytokines, functional iron deficiency develops, accompanied by hypoferremia with normal or elevated serum ferritin, heme synthesis is disrupted, and erythropoiesis decreases [4, 5].

Spondyloarthritis (SpA) is a chronic autoimmune disease characterized by persistent systemic inflammation, damage to the axial skeleton, entheses, and peripheral joints, and possible extra-articular manifestations, such as psoriasis, uveitis, Crohn's disease or ulcerative colitis [6]. SpA is highly prevalent in the general population, but currently there is no convincing evidence of the incidence and features of anemia in this category of patients. Proper identification of the pathogenetic type of anemia contributes to the choice of the optimal therapeutic strategy for correcting anemia in SpA. The presence of ACD is considered as a marker of the activity of systemic inflammation, its severity and pathogenetic features may influence the choice of backbone antirheumatic therapy, determining a personalized approach to SpA treatment. Therefore, it is of interest to study the relationship between anemia and inflammation in SpA.

The aim of the study was to evaluate the relationship between hemoglobin levels and markers of SpA activity, such as serum concentrations of TNF-α, IL-6, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR).

MATERIALS AND METHODS

The study included 92 patients with SpA (55 males and 37 females aged 42.9 ± 11.6 years, SpA duration – 14.8 ± 9.6 years), who were admitted to the Department of Rheumatology at Saratov Regional Hospital from 2017 to 2019. Inclusion criteria were the following: people aged 18 years and older, a confirmed diagnosis of SpA according to the Assessment of Spondyloarthritis International Society (ASAS) classification criteria (2009) [7], a signed informed consent to participate in the study. Exclusion criteria included therapy with biological disease-modifying antirheumatic drugs (bDMARDs); true iron deficiency, posthemorrhagic, megaloblastic or hemolytic anemia; cancers; tuberculosis, HIV, HBV and HCV infections; stage 3–5 chronic kidney disease; pregnancy and lactation.

The study was carried out according to the principles set out in the Declaration of Helsinki and approved by the local Ethics Committee at V.I.Razumovsky Saratov State Medical University (Protocol No. 3 of 07.11.2017).

The SpA activity was determined by calculating the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Ankylosing Spondylitis Disease Activity Score with CRP (ASDAS-CRP). Complete blood count (CBC) and serum biochemistry parameters, including iron status and CRP, were evaluated. Serum TNF-α and IL-6 levels were measured in all patients by enzyme-linked immunosorbent assay (ELISA) using alpha-TNF-EIA-BEST and Interleukin-6-EIA-BEST reagent kits (Vector-Best, Russian Federation). According to the instructions, the reference concentrations were TNF-α < 6 pg / ml and IL-6 < 10 pg / ml.

Anemia was defined at a hemoglobin concentration of < 130 g / l for men and < 120 g / l for women. ACD was diagnosed when serum ferritin was > 100 ng / ml, transferrin saturation (TSAT) was < 20%, and CRP was > 5 mg / l. Combined ACD / IDA was diagnosed when serum ferritin was 30–100 ng / ml, TSAT was < 20%, and CRP was > 5 mg / l [8].

Statistical processing of the data was performed using SPSS version 26.0 software (IBM SPSS Statistics, USA). Normality of sample distribution was examined by the Shapiro –Wilk and Kolmogorov – Smirnov tests, the distribution was considered

normal at $p > 0.05$. Normally distributed, quantitative variables were presented as the mean and standard deviation ($M \pm SD$). Variables characterized by non-normal distribution were presented as the median and the upper and lower quartiles Me [Q_1; Q_3]. Depending on the type of data distribution, parametric and non-parametric methods were used. To compare the differences between quantitative variables in two independent groups, the Student's t-test was used for normally distributed variables, and the Mann – Whitney test was used for non-normally distributed variables. To assess the differences between the categorical variables, the Pearson's chi-square test χ^2 or Fisher's exact test was used. The odds ratio (OR) with 95% confidence interval (CI) was used for assessing the effect of cytokine overproduction on the development of anemia in patients with SpA. Relationships between variables were tested using the Spearman's rank correlation coefficient. The Chaddock scale was used to determine the strength of the relationship. The differences were considered statistically significant at $p < 0.05$; $p < 0.1$ was considered as a trend toward a difference.

RESULTS

Anemia was detected in 52 (57%) patients included in the study: ACD was found in 13 (25%) patients, combined ACD / IDA was detected in 39 (75%) patients. In 47 (90%) patients, the severity of the disease was mild, in 5 (10%) patients, the severity of anemia was moderate. SpA patients with and without anemia were comparable in gender, age, disease duration, and SpA activity, and according to the BASDAI and ASDAS-CRP scores, the majority of patients had high disease activity (Table 1). In SpA patients with anemia, a trend toward more frequent use of multiagent therapy, including non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and synthetic disease-modifying antirheumatic drugs (DMARDs), was observed. Among SpA patients without anemia, in most cases (70%), NSAID therapy was prescribed without the additional use of glucocorticoids, and synthetic DMARDs were used in 40% of patients (Table 1).

The main CBC and iron status parameters and markers of systemic inflammation in SpA patients included in the study are shown in Table 2. Anisocytosis and a trend toward microcytosis and hypochromia of red blood cells were detected according to the obtained values in the patients with anemia. These patients were characterized by a higher platelet count, reduced TSAT, and a trend toward a decrease in serum iron, which is typical of ACD. A trend toward an increase in serum IL-6 concentrations and a significant increase in CRP and ESR values were identified in anemic patients compared with patients without anemia ($p = 0.051$, $p = 0.001$, and $p < 0.001$, respectively). Nevertheless, no significant differences in the TNF-α concentrations in patients with and without anemia were revealed ($p = 0.245$).

Table 1

Main demographic and clinical characteristics of SpA patients

Parameter	All patients with SpA ($n = 92$)		p
	Without anemia ($n = 40$)	With anemia ($n = 52$)	
Age, years, M SD	45.113.0	41.310.3	0.139
Males, n (%)	25 (63)	30 (58)	0.641
Disease duration, years, Me [Q_1–Q_3]	12.3 [7.4–20.3]	15.8 [8.7–21.6]	0.521
BASDAI, M SD	4.82.2	5.62.1	0.117
BASDAI > 4, n (%)	22 (55)	33 (64)	0.799
ASDAS-CRP, M SD	3.31.0	3.81.2	0.062
ASDAS-CRP 2.1, n (%)	27 (68)	42 (81)	0.434
SpA treatment, n (%):	–	–	–
NSAIDs	28 (70)	26 (50)	0.053
NSAIDs + glucocorticoids	11 (28)	24 (46)	0.068
Synthetic DMARDs, including:	16 (40)	31 (60)	0.103
Methotrexate	8 (50)	14 (45)	0.472
Sulfasalazine	7 (44)	12 (39)	0.608
Methotrexate + sulfasalazine	1 (6)	4 (13)	0.383
Hydroxychloroquine	–	1 (3)	–

A correlation analysis was performed to determine the relationship between markers of systemic inflammation, disease activity scores, and hemoglobin levels (Table 3). The hemoglobin was inversely correlated with CRP and ESR ($p = 0.001$ and $p < 0.001$, respectively). A positive correlation between serum IL-6 concentration and other markers of systemic inflammation, such as TNF-α, CRP, and ESR, was observed ($p < 0.001$ for all).

The analysis of the frequency of anemia depending on excess serum levels of TNF-α and IL-6 is presented in Table 4. The odds for anemia were 5.3 times higher with elevated IL-6 concentration > 10 pg / ml (95% CI: 1.4–19.9). Moderate strength of the relationship between the compared features was noted (Cramer's V = 0.281).

Table 2

Hematological and ferrokinetic parameters and systemic inflammatory markers in patients with SpA

Parameter	All patients with SpA (n = 92)				p
	without anemia (n = 40)		with anemia (n = 52)		
	n	Me [Q_1–Q_3]	n	Me [Q_1–Q_3]	
Hemoglobin, g / l	40	139 [134–149]	52	116 [107–120]	<0.001*
Mean corpuscular volume (MCV), fl	40	90.4 [88.9–92.9]	52	85.3 [79.3–91.0]	0.002*
Mean corpuscular hemoglobin (MCH), pg	40	30.3 [29.5–30.9]	52	27.3 [24.6–29.7]	<0.001*
Red cell distribution width (RDW), %	40	13.5 [12.9–14.3]	52	15.1 [13.6–16.8]	<0.001*
Platelets, 10^9 / l	40	278 [236–319]	52	297 [265–362]	0.028*
ESR, mm / h	40	10 [6–15]	52	23 [12–32]	<0.001*
Serum iron, μmol / l	16	13.8 [10.5–14.0]	52	8.7 [6.3–13.7]	0.071
Ferritin, ng / ml	16	69 [49–162]	52	70 [46–98]	0.640
Transferrin, g / l	16	2.2 [2.0–2.3]	52	2.2 [2.1–2.3]	0.688
TSAT, %	16	24.9 [18.0–27.5]	52	15.7 [11.2–23.0]	0.038*
CRP, mg / l	40	9.0 [3.9–16.6]	52	17.8 [9.5–30.5]	0.001*
TNF-α, pg / ml	40	3.0 [2.4–4.0]	52	3.4 [2.7–4.1]	0.245
IL-6, pg / ml	40	4.1 [2.0–6.7]	52	5.4 [2.4–11.7]	0.051

* $p < 0.05$

Table 3

Correlation analysis of systemic inflammatory markers and clinical and laboratory parameters in patients with SpA

Parameter	CRP	ESR	IL-6	TNF-α
BASDAI	r = 0.147 p = 0.202	r = 0.105 p = 0.362	r = –0.094 p = 0.417	r = 0.102 p = 0.378
ASDAS-CRP	r = 0.489 p < 0.001*	r = 0.358 p = 0.001*	r = 0.160 p = 0.167	r = 0.208 p = 0.071
Hemoglobin	r = –0.327 p = 0.001*	r = –0.527 p < 0.001*	r = –0.155 p = 0.142	r = –0.104 p = 0.323
TNF-α	r = 0.153 p = 0.146	r = 0.233 p = 0.025*	r = 0.431 p < 0.001*	1
IL-6	r = 0.361 p < 0.001*	r = 0.369 p < 0.001*	1	r = 0.431 p < 0.001*

* $p < 0.05$

Table 4

Comparison of the frequency of anemia depending on increased serum concentrations of TNFα and IL6

Risk factor	Frequency of anemia		p	OR (95% CI)
	The presence of the factor, n (%)	The absence of the factor, n (%)		
TNF-α > 6 pg / ml	4 (80.0)	48 (55.2)	0.383	3.3 (0.3–30.3)
IL-6 > 10 pg / ml	16 (84.2)	36 (50.0)	0.009*	5.3 (1.4–19.9)

* $p < 0.05$

DISCUSSION

In this study, we attempted to assess the contribution of systemic inflammation to the development of anemia in patients with SpA. No significant differences in the TNF-α levels in patients with and without anemia were revealed. At the same time, a trend toward an increase in the serum IL-6 concentrations in patients with reduced hemoglobin levels was observed, and overproduction of this cytokine caused a more than five-fold increase in the odds for developing anemia. The traditional inflammatory markers, CRP and ESR, were significantly higher in SpA patients with anemia, and these parameters were correlated with ASDAS-CRP score, hemoglobin level, and serum IL-6 concentration.

It was found that serum IL-6 concentration in patients with SpA was significantly higher than in healthy individuals [9, 10]. However, according to the results of clinical trials reported by J. Sieper et al. [11, 12], the efficacy of IL-6 inhibitors (tocilizumab, sarilumab) in the treatment of ankylosing spondylitis (AS) has not been confirmed. Despite a significant decrease in the laboratory markers of disease activity, no improvement in symptoms related to the axial skeleton and peripheral joints in patients and no significant differences in the ASAS20 and ASAS40 response rate compared with the control group were identified. It is worth noting that these studies did not analyze the relationships between hemoglobin and IL-6 concentrations and did not evaluate the hematological response to treatment with IL-6 inhibitors. At the same time, IL-6 inhibitors are preferred in patients with rheumatoid arthritis who have anemia and other signs of IL-6-dependent inflammation due to their proven high clinical efficacy [13]. It should be assumed that the use of IL-6 inhibitors in SpA patients with severe ACD may be justified.

Anti-TNF-α therapy in patients with psoriatic arthritis [14] and AS [15] with isolated ACD without signs of true iron deficiency led to a significant increase in hemoglobin levels and a decrease in serum CRP and ferritin concentrations. It is known that cytokines are able to potentiate the effects of each other, especially this is typical of the triad of inflammatory mediators – TNF-α, IL-1β, and IL-6. The opposite effect is also realized – TNF-α blockade in the cytokine ensemble can reduce IL-6 expression playing a key role in the development of ACD.

Long-term use of NSAIDs as first-line drug treatment is recommended in patients with axial SpA. Y. Yan et al. in their study [16] demonstrated a significant decrease in laboratory inflammatory markers in AS patients treated with NSAIDs: statistically significant reduction in the levels of ESR, CRP, IL-6, and TNF-α was shown. Taking into account the pathogenesis of anemia, it is obvious that appropriate anti-inflammatory therapy with NSAIDs can prevent the development of anemia in patients with SpA. On the other hand, continuous NSAID requires monitoring of potential side effects, especially developing drug-induced gastrointestinal bleeding and IDA. In this study, 75% of patients were diagnosed with combined ACD / IDA, but had no signs of NSAID-induced enteropathy. Previously, there were no data confirming the association between IDA and the use of NSAIDs in patients with SpA [17]. The presence of iron deficiency in anemia should be considered as a consequence of hepcidin-induced alteration of iron homeostasis under the conditions of chronic systemic inflammation.

The results of this study confirm the existence of a relationship between the activity of systemic inflammation and hemoglobin levels in patients with SpA. Taking into account the above, correction of anemia in patients with SpA should be based on suppression and, if impossible, minimization of disease activity, which is the immediate therapeutic goal, provided appropriate basic antirheumatic therapy is chosen.

CONCLUSION

The study confirmed the relationship between the activity of systemic inflammation and anemia in patients with SpA. The majority of patients had mixed anemia (ACD / IDA) with a slight or moderate decrease in hemoglobin levels. Inflammation should be considered as the main component in the development of anemia. Taking into account the pathogenesis of ACD, the basis of antianemic therapy is to achieve remission, and if impossible, minimal SpA activity. Additional studies are required to determine the effect of NSAIDs and synthetic and biological DMARDs on the development and course of anemia in patients with SpA.

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Platelet aggregation under the conditions of vortex flow *in vitro* in patients with chronic heart failure

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ABSTRACT

Aim. To compare the effect of increased concentrations of aggregation inducers (five-fold addition) under standard conditions and under the conditions of vortex flow *in vitro* on platelet aggregation in patients with chronic heart failure (CHF).

Materials and methods. The study included 28 patients. The activity of platelet aggregation in platelet-rich plasma (PRP) was evaluated according to light transmission curves (%) and the average size of aggregates (relative units (rel. units)). The aggregation inducer was added once at 10 seconds of the study (standard procedure) and five times at 10 seconds, 1, 2, 3, and 4 minutes of the study with a constant stirring rate of 800 rpm. The same parameters were evaluated under the conditions of vortex flow, which was created by changing the stirring rate of the PRP from 800 rpm to 0 rpm and again to 800 rpm by pressing the centrifugation button on the analyzer.

Results. In the course of the study, the size of the aggregates increased in patients with CHF only under the conditions of vortex flow. When a collagen aggregation inducer was added both at the concentration of 2 mmol / l and 10 mmol / l, platelet aggregation parameters increased under the conditions of vortex flow. During the study of epinephrine-induced platelet aggregation in patients with CHF, an increase in the aggregation parameters was revealed, both at five-fold addition of the inducer and under the conditions of vortex flow compared with the standard method.

Conclusion. The proposed methodological approaches to creating the conditions for vortex flow *in vitro* and to five-fold addition of epinephrine showed an increase in the size of the aggregates and the degree of platelet aggregation. Collagen-induced aggregation under the conditions of vortex flow revealed 7 (25%) patients with high residual platelet reactivity (HRPR), and epinephrine-induced aggregation detected 15 (54%) patients with HRPR.

Keywords: aggregation, platelets, collagen, epinephrine, chronic heart failure, residual reactivity

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

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Агрегация тромбоцитов в условиях «вихревого» потока *in vitro* у пациентов с хронической сердечной недостаточностью

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РЕЗЮМЕ

Цель – сравнительное изучение влияния повышенной концентрации индукторов агрегации при пятикратном добавлении в стандартных условиях и в условиях «вихревого» потока *in vitro* на агрегацию тромбоцитов у пациентов с хронической сердечной недостаточностью.

Материалы и методы. В исследование включены 28 пациентов. Активность агрегации тромбоцитов в богатой тромбоцитами плазме (БТП) оценивали по кривым светопропускания (%) и среднего размера агрегатам (в относительных единицах, отн. ед.). Определение проводили с индуктором агрегации при однократном добавлении на 10-й с исследования (стандартная методика) и при пятикратном добавлении индуктора на 10-й с, 1, 2, 3 и 4-й мин исследования, при постоянном перемешивании со скоростью 800 об/мин. Эти же параметры оценивали в условиях «вихревого» потока плазмы, что достигалось изменением скорости перемешивания БТП с 800 до 0 об/мин и вновь до 800 об/мин с помощью кнопки выключения и включения центрифугирования на анализаторе.

Результаты. В ходе проведенного исследования у пациентов увеличился размер агрегатов только в условиях «вихревого» потока. При добавлении индуктора агрегации коллагена как в концентрации 2 ммоль/л, так и 10 ммоль/л показатели агрегации тромбоцитов увеличились в условиях «вихревого» потока. В ходе исследования эпинефрин-индуцированной агрегации тромбоцитов у пациентов выявили возрастание параметров агрегации, как при пятикратном добавлении индуктора, так и в условиях «вихревого» потока по сравнению со стандартной методикой.

Заключение. Предложенные нами методические подходы по созданию условий «вихревого» потока *in vitro* и по пятикратному добавления индуктора эпинефрина показали увеличение размеров агрегатов и степени агрегации тромбоцитов. Коллаген-индуцированная агрегация в условиях «вихревого» потока позволила выявить 7 (25%) пациентов с высокой остаточной реактивностью тромбоцитов, а эпинефрин-индуцированная – 15 (54%) пациентов.

Ключевые слова: агрегация, тромбоцит, коллаген, эпинефрин, хроническая сердечная недостаточность, остаточная реактивность

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

Источник финансирования. Исследование выполнено в рамках поисковой темы НИИ кардиологии АААА-А20-120041090007-8.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом НИИ кардиологии Томского НИМЦ (протокол № 139 от 18.11.2015).

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INTRODUCTION

Cardiovascular diseases (CVDs) are the main cause of morbidity worldwide, while chronic heart failure (CHF) is a progressive disabling condition with a high mortality rate. Despite advances in treatment, patients with CHF have an increased risk of thrombosis. The causes of thrombosis in patients with CHF include impaired hemodynamics, changes in rheological properties of the blood, coagulation disorders, and increased platelet activity. Platelets are crucial for the development of blood clots in blood vessels [1, 2]. In case of vascular le-

sions, pathological activation of platelets may occur, leading to uncontrolled growth of a blood clot, which causes subsequent ischemic events. High residual platelet reactivity (HRPR) in patients is associated with the development of ischemic events, which has been proven in numerous studies and meta-analyses [2–6].

The mechanisms of platelet aggregation are investigated by standard methods without taking into account turbulence or vortex flow. However, in cardiovascular pathology, the rheology of the blood changes, and vortex flow has strong prothrombotic effects. The adhesion of several platelets creates a snowball effect, inducing pronounced platelet aggregation with high residual reactivity, which results in rapid vessel occlusion. It is known that the gold standard and the most accessible method for assessing platelet aggregation is light transmission aggregometry (LTA), which analyzes aggregation by light transmission curves and aggregate sizes [6]. However, the sensitivity of the methods currently used in routine practice for assessing residual platelet aggregation is insufficient [7]. Therefore, there is a need to search for new promising methods for the diagnosis of increased proaggregant potential of platelets for preventing thrombosis. Thus, the study of platelet aggregation in patients with CVDs under the conditions of vortex flow in platelet-rich plasma (PRP) is relevant. Knowledge in this research area is relevant both for clinical and fundamental medicine, as well as for development of new diagnostic methods.

The aim of the study was to compare the effect of increased concentrations of aggregation inducers (five-fold addition) under standard conditions and under the conditions of vortex flow *in vitro* on platelet aggregation in patients with CHF.

MATERIALS AND METHODS

A single-stage cross-sectional study was conducted. Recruitment of patients was carried out at the Cardiology Research Institute upon planned hospitalization in the Department of Myocardial Pathology (under the guidance of Professor A.A. Garganeeva) in accordance with the principles of the Declaration of Helsinki. The study included 28 patients aged 41–83 years (18 men and 10 women). Inclusion criteria: stable coronary artery disease in combination with functional class (FC) I–III CHF according to the New York Heart Association classification and continuous use of antiplatelet therapy for 6 months. All the examined patients received regular combination therapy in accordance with modern recommendations for the treatment of coronary artery disease with comorbid CHF. Laboratory and instrumental research methods were used in all patients in accordance with the recommendations for the diagnosis and treatment of coronary artery disease with comorbid CHF. Exclusion criteria: non-adherence to therapy; acute vascular complications which occurred no later than 6 months ago; severe concomitant pathology; clinical and laboratory signs of acute inflammation; atrial fibrillation; high-grade ventricular arrhythmia according to the Lown grading system; and refusal to participate in the study.

A special study on assessing platelet aggregation was carried out using the Born method as modified by Z.A. Gabbasov on a laser two-channel analyzer (220 LA, Biola Scientific, Russian Federation). To isolate a suspension of human platelets, peripheral venous blood was used, collected in the morning on an empty stomach in a 7 ml vacuum tube with 3.8% sodium citrate as an anticoagulant, with a 6:1 blood / anticoagulant ratio.

Experimental values of light transmission were determined for each patient's blood sample, where platelet-poor plasma is taken as 0%, and platelet-rich plasma is taken as 100% aggregation in this patient. Platelet aggregation activity in platelet-rich plasma was evaluated by light transmission curves (%) and average size of aggregates (in relative units (rel. units)). The aggregation inducer was added once at 10 seconds of the study (standard procedure) and five times at 10 seconds, 1, 2, 3, and 4 minutes of the study with a constant stirring rate of 800 rpm. The same parameters were evaluated using the method for studying platelet aggregation under the conditions of vortex flow, which was created by a five-time change in the stirring rate of platelet-rich plasma from 800 rpm to 0 rpm and again to 800 rpm, with five-fold centrifugation on the analyzer for 10 seconds, 1, 2, 3, and 4 minutes with a delay of 10 seconds. The five-fold addition of the inducer and creation of vortex flow conditions with changes in the stirring rate were selected experimentally. Collagen and epinephrine (Helena, Great Britain) were used as natural inducers at concentrations of 2 mmol / l (standard procedure) and 10 mmol / l (with five-fold addition of 2 mmol / l of the inducer at 10 seconds, 1, 2, 3, and 4 minutes of the study).

Statistical data processing was carried out using SPSS (version 19) and Statistica 10.0 software packages. The Shapiro – Wilk test was used to assess the distribution of quantitative variables. The distribution

of quantitative aggregation parameters did not follow normal distribution; the aggregation data were presented as the median and the interquartile range *Me* (Q_1; Q_3). The significance of the differences for paired or dependent samples was evaluated using the Wilcoxon T-test. The differences between the samples were considered statistically significant at $p < 0.05$.

RESULTS

Among the recruited individuals, patients with functional class III angina pectoris prevailed (15 (53%) patients); functional class II was established in 11 (30%) cases, functional class I – in 2 (7%) cases. In the anamnesis, 8 (29%) patients had a Q-wave myocardial infarction (MI) ≥ 6 months before. In most cases, patients included in the study were diagnosed with multivessel coronary artery disease (22 (79%) patients). Cardiovascular risk factors were also widespread among the patients: smoking – in 17 (61%) patients, overweight and obesity – in 22 (78%) patients, arterial hypertension – in 20 (71%) patients, dyslipidemia – in 13 (46%) patients, type 2 diabetes – in 13 (46%) patients.

In the course of the study in patients with CHF, spontaneous platelet aggregation indices determined by the standard method were 3.1 (1.5; 4.0) % and 1.7 (1.1; 2.0) relative units. Under the conditions of vortex flow, only the size of the aggregate significantly increased to 5.4 (3.2; 6.1) relative units ($p = 0.04$). The size of the aggregates in patients with cardiovascular pathology should not exceed 4 relative units; an increase in the parameter indicates HRPR. When assessing spontaneous platelet aggregation in vortex flow in terms of the aggregate size, 5 (18%) patients with HRPR were identified among the CHF patients.

The degree of platelet aggregation and the size of the aggregates in patients with CHF with the addition of collagen at a concentration of 2 mmol / l (standard procedure) were 9.3 (2.1; 65.4) % and 3.1 (1.9; 10.1) relative units, respectively. When the stirring rate changed from 800 rpm to 0 and then to again 800 rpm, the aggregation rate significantly increased to 47.9 (40.6; 95.0)% ($p = 0.00$), and the size of the aggregates was 3.8 (1.3; 10.7) relative units. However, these parameters did not exceed the reference values (up to 50% for the degree of aggregation and up to 4.5 relative units for the size of aggregates).

Under the conditions of five-fold addition of collagen at 10 seconds, and 1, 2, 3, and 4 minutes of the study, no significant changes were observed, and the parameters were 22.3 (17.3; 89.6)% for the degree of aggregation and 6.42 (2.1; 39.8) relative units for the size of the aggregates. Under the conditions of vortex flow, the parameters of collagen-induced platelet aggregation increased, the degree of aggregation amounted to 80.1 (13.5; 165.0) % ($p = 0.04$), and the size of the aggregates increased to 32.9 (1.1; 43.7) ($p = 0.00$) relative units. According to the results of the study on collagen-induced aggregation in patients with CHF under the conditions of vortex flow, 7 (25%) patients with HRPR were identified, whereas with five-fold addition of the inducer, no significant differences were identified compared with the standard method.

The parameters of standard epinephrine-induced platelet aggregation in patients with CHF at the inducer concentration of 2 mmol / l were 46.7 (35.8; 66.2)% for the degree of aggregation and 15.0 (11.4; 18.9) relative units for the size of the aggregates. With a change in the stirring speed (800 rpm – 0 rpm – 800 rpm), the parameters significantly increased to 52.7 (41.3; 76.5)% ($p = 0.00$) and 19.4 (17.3; 20.6) ($p = 0.04$) relative units, respectively.

Under the conditions of five-fold addition of epinephrine, only the degree of aggregation significantly increased and amounted to 52.5 (41.9; 74.5)% ($p = 0.03$), while the size of the aggregates remained unchanged and amounted to 15.8 (12.2; 18.4) relative units ($p = 0.02$). Under the conditions of vortex flow, the parameters of epinephrine-induced aggregation significantly increased. The degree of aggregation was 75.4 (62.0; 80.5)% ($p = 0.04$), while the increase in the size of the aggregates was multiple and reached 356.0 (230.5; 462.5) relative units ($p = 0.03$). Thus, during the study of epinephrine-induced platelet aggregation in patients with CHF, we revealed an increase in aggregation parameters, both with five-time addition of the inducer, and under the conditions of vortex flow compared with the standard method. As a result of the study, HRPR was detected in 15 (54%) patients, which was the largest number of detected HRPR cases among all the methodological approaches used.

DISCUSSION

Significant changes in platelet aggregation parameters obtained during the study, with five-fold addition of the inducer and under the conditions of vortex flow, compared with the standard technique, indicate the need to study new methodological approaches to assessing platelet aggregation and identifying patients with residual platelet reactivity to prevent possible ischemic events in CHF patients. Modern therapy in

a hospital setting is very expensive, so the search for simple and inexpensive diagnostic tests is becoming more relevant. Discussion about the relevance of studying platelet aggregation in patients with CHF is still going on, which determines the need for further research in this area [5, 8, 9].

The present work was an open, single-center, cross-sectional study. We have shown that standard methods for studying platelet aggregation are not always sufficient to identify HRPR. The use of increased concentrations of epinephrine with five-fold addition during the research at 10 seconds, 1, 2, 3, and 4 minutes of the study and the use of spontaneous, collagen- and epinephrine-induced platelet aggregation under the conditions of vortex flow with a change in the stirring rate (800rpm – 0rpm – 800rpm) increase the accuracy of aggregation assessment in detecting HRPR in patients with CHF. The results of several independent meta-analyses involving more than 10,000 patients showed that HRPR was associated with a significant increase in the incidence of MI, stent thrombosis, and death from CVDs [1, 2, 4].

Blood circulating in the vessels under the conditions of a pressure drop impacts blood cells and blood vessel walls [10]. The blood flow is laminar, with a maximum velocity in the center of the vessel lumen and zero velocity at the vessel wall [11]. Biomechanical forces created by the blood pressure are crucial for aggregation or separation of the main components involved in blood clotting. Within normal hematocrit levels (~ 40%), erythrocytes mainly circulate along the central axis of the blood vessel due to axial migration. Consequently, platelets move in close proximity to the vessel walls, which facilitates their binding to adhesive ligands in the reactive endothelial layer in damaged regions of the vessel [11–13]. Under physiological conditions, the flow of blood in large arteries is laminar, but arterial stenosis due to atherosclerotic lesions or pre-existing blood clots can alter the blood flow [8, 10, 14, 15].

Over decades of study, it has been established that platelets are crucial for blood clot formation in healthy and pathologically altered blood vessels. When the integrity of the vessels is impaired, circulating platelets linger at the site of injury, where they aggregate, forming hemostatic thrombi, thereby preventing further bleeding. However, under the conditions of turbulence or vortex flow, transient aggregates are formed without prior activation or shape change, which stabilizes discoid platelet aggregates and leads to uncontrolled growth of unstable and weakly adhered thrombi, which, in turn, can clog a blood vessel or embolize it, causing subsequent ischemic events [16].

The molecular processes that cause pathological blood clotting are in many ways similar to the processes that control physiological blood clotting. The biggest problem of antiplatelet therapy is differentiation between pathological and physiological platelet responses. Currently, antiplatelet drugs that are available on the market are not effective against targeted pathological blood clots without impairment of normal hemostasis. Transition from laminar to vortex blood flow, occurring in the bloodstream, leads to platelet aggregation in atherosclerosis. The turbulent blood flow in clogged blood vessels can be two times faster than in healthy vessels. The use of vortex flow in the microenvironment of a blood clot allows to differentiate between thrombosis and physiological hemostasis and develop selective antiplatelet therapy.

It should be noted that modern antiplatelet therapy fails to selectively prevent pathological thrombosis without interfering with the physiological hemostasis. One of the significant differences between these two processes is the difference in the types of blood flow in the vessels, and the pathological turbulent blood is identified in pathological blood clots at the sites of vascular occlusion or atherosclerotic plaque rupture [17]. It is known that no existing, clinically used antiplatelet drug is able to specifically respond to this biomechanical force at the site of pathological thrombosis. Aspirin, which is the gold standard for antiplatelet therapy, cannot completely inhibit platelet aggregation, as shown in numerous studies [18, 19]. In pathological blood flow, selective inhibition of cyclooxygenase-2 can even enhance platelet aggregation by reducing basal production of prostacyclin (prostaglandin I2; PGI 2), a powerful inhibitor of platelet aggregation [20, 21]. To date, there are no methods that would take into account these changes in the blood flow. Analysis of platelet aggregation by the aggregate size curve and the degree of aggregation under conditions of vortex flow proves the possibility of detecting HRPR in patients with CHF, taking into account changes in the blood flow.

The comparison of the methods showed that five-fold addition of the inducer and creation of vortex flow by changing the stirring rate make it possible to identify platelets with high residual activity and a tendency to form large aggregates. From our point of view, identification of HRPR by new methodological approaches will allow to determine increased cardiovascular risk in patients with CHF.

Limitations of the study include its single-stage design and a relatively small number of examined patients. However, the results obtained prove the need for further studies on investigating platelet aggregation and identifying HRPR in patients in order to improve techniques and prevent cardiovascular events.

CONCLUSION

Standard methods for studying platelet aggregation are not always sufficient to detect an increased proaggregant potential of platelets, which is important for the diagnosis and prevention of cardiovascular complications. The proposed methodological approaches to creating the conditions for vortex flow *in vitro* and to five-fold addition of epinephrine in collagen-induced and epinephrine-induced aggregation showed an increase in the size of the aggregates and the degree of platelet aggregation, which proves the prospects of these methodological approaches for detecting HRPR in patients with CHF.

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Authors contribution

Trubacheva O.A., Kologrivova I.V. – conception and design, carrying out of the experimental part of the study, analysis and interpretation of the data, drafting of the article. Suslova T.E., Garganeeva A.A. – justification of the manuscript, final approval of the manuscript for publication. Swarovskaya A.V. – interview and selection of patients, carrying out of the necessary examinations.

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Insulin-like growth factors and their transporter proteins in the liver of rats with experimental diabetes, adenocarcinoma of the uterine corpus, and their combination

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ABSTRACT

Aim. To investigate the content of insulin-like growth factor (IGF)-1, IGF-2, and their transporter proteins IGFBP-1 and IGFBP-2 in the liver of rats with experimental diabetes, Guerin's carcinoma, and their combination.

Materials and methods. The experiment was carried out on 64 white outbred rats of both sexes, which were divided into 4 groups of 8 animals each: group 1 – intact animals, group 2 – animals with experimental diabetes, group 3 – animals with subcutaneously inoculated Guerin's carcinoma, group 4 – animals with experimental diabetes and subcutaneously inoculated Guerin's carcinoma. In the study, biochemical and statistical analyses and enzyme immunoassays were performed.

Results. In the liver of the outbred rats, sex specificity in the content of insulin-like growth factors and IGFBP-1 was established: the levels of IGF-1, IGF-2, and IGFBP-1 in males were lower than in females. It was shown that the development of diabetes mellitus and the growth of Guerin's carcinoma led to changes in the sex-specific components in the rat liver.

Conclusion. The growth of Guerin's carcinoma and the progression of diabetes mellitus cause multidirectional changes in IGF and IGFBP levels in the liver of females and unidirectional changes in the liver of males. Following the growth of Guerin's carcinoma against the background of diabetes mellitus, sex-specific differences in the content of the studied parameters were minimized. It was shown that diabetes mellitus changed the metabolic profile of the liver in the animals of both sexes.

Keywords: Guerin's carcinoma, diabetes mellitus, liver, IGF, IGFBP

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

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Инсулиноподобные факторы роста и их белки-переносчики в печени крыс при экспериментальном диабете, злокачественном росте аденокарциномы тела матки и их сочетании

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РЕЗЮМЕ

Цель. Исследовать содержание инсулиноподобных факторов роста (IGF) 1 и 2, их белков-переносчиков IGFBP-1 и IGFBP-2 в печени крыс с сахарным диабетом, аденокарциномой Герена и при их сочетании.

Материалы и методы. Эксперимент проводился на 64 белых беспородных крысах обоего пола, которые были разделены на 4 группы по 8 особей: 1-я – интактные животные, 2-я – животные с экспериментальным диабетом, 3-я – животные с подкожной перевивкой карциномы Герена, 4-я – животные с экспериментальным диабетом и с подкожной перевивкой опухоли Герена. В работе осуществляли биохимический, иммуноферментный и статистический анализы.

Результаты. В печени беспородных крыс установлена половая специфичность содержания инсулиноподобных факторов роста и IGFBP-1: у самцов уровень IGF-1, IGF-2 и IGFBP-1 оказался ниже, чем у самок. Развитие сахарного диабета и рост перевивной карциномы Герена в самостоятельных вариантах приводили к изменению половых особенностей изученых компонентов в печени крыс.

Заключение. Рост карциномы Герена и сахарный диабет вызывают разнонаправленные изменения IGF и IGFBP в печени самок, но однонаправленные – в печени самцов. В результате развития карциномы Герена на фоне сахарного диабета половые различия в содержании изученных показателей нивелируются, сахарный диабет изменяет метаболический профиль печени у животных обоего пола.

Ключевые слова: карцинома Герена, сахарный диабет, печень, IGF, IGFBP

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

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INTRODUCTION

The incidence of diabetes mellitus (DM) and cancer has increased significantly in recent years. In addition, there are many common risk factors for both DM and cancer. Much epidemiological evidence indicates that DM is considered an independent risk factor for increased incidence of different cancers and death from them. Morbidity and mortality from various types of cancer, such as cancer of the pancreas, liver, colon, breast, endometrium, and bladder, are slightly increased in DM patients. Although the underlying biological mechanisms are not fully understood, studies have confirmed that the growth hormone / insulin-like growth factor (IGF) 1 axis, hyperglycemia, and sex hormones create favorable conditions for cancer cell proliferation and metastasis. The growth hormone / IGF-1 axis activates several metabolic and mitogenic signaling pathways; hyperglycemia provides energy for the growth of cancer cells. Thus, these factors affect all cancers, while sex hormones play an important role only in breast cancer, endometrial cancer, and prostate cancer [1].

The most common types of DM are type 1 and type 2 diabetes. On the one hand, an autoimmune disorder of insulin-producing beta cells causing absolute insulin deficiency leads to type 1 diabetes mellitus (T1DM) and accounts for about 5% from 10% of all DM cases. On the other hand, type 2 diabetes mellitus (T2DM) is associated with metabolic disorders in which cells become insensitive to insulin and, therefore, exhibit relative insulin deficiency. Several studies have shown that although both T1DM and T2DM are associated with higher risks of cancer, T2DM has a stronger association with cancer, both epidemiologically and biologically [2, 3].

Endometrial cancer (EC) is the most common gynecologic cancer. Compared with other cancers, EC is often diagnosed earlier and has a better prognosis. However, mortality from it has increased significantly over the past 20 years. An association between DM and EC has been consistently confirmed by cohort studies and meta-analyses [4]. In addition, a systematic review and meta-analysis summarized results of 29 cohort studies and found the incidence of EC in women with and without DM. The cumulative relative risk was 1.89 (95% confidence interval (CI): 1.46–2.45; $p < 0.001$), and the cumulative incidence rate was 1.61 (95% CI: 1.51–1.71; $p < 0.001$), which again confirms that DM is an independent risk factor for higher incidence of EC [5].

Insulin is a peptide hormone that regulates the metabolism of carbohydrates and fats and improves glucose absorption. Insulin loses the function of increasing cellular uptake and utilization of glucose in DM patients, which is clinically defined as insulin resistance [6]. High insulin levels are a sign of hyperinsulinemia, which stimulates liver cells to produce IGF-1, when insulin binds to the insulin receptor on the surface of target cells. IGF-1 binds to the IGF-1 receptor (IGF-1R), receptor tyrosine kinase, to activate several metabolic and mitogenic signaling pathways that regulate cancer cell proliferation, differentiation, and apoptosis [7].

Insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) are fundamental mediators of cell growth, development, and survival and are expressed in most tissues. Epidemiological data indicate the relationship of IGF and IGFBP with the risk of prostate, breast, colorectal, and thyroid cancers [8]. DM patients show higher IGF-1 levels, which makes them more susceptible to an increased risk of many cancer types. In addition, many studies have demonstrated that IGF-1 is more often expressed in cells of hormone-dependent cancer than in other types of cancer [9].

An experiment on a mouse model of HER2-mediated breast cancer confirmed that hyperinsulinemia promotes enhanced growth of breast tumors through the growth hormone / IGF-1 axis [10]. Epidemiological evidence suggests that DM is associated with a higher risk of development and death from many cancer types. The underlying mechanisms linking DM and cancer are not yet fully understood; however, hyperglycemia as a sign of DM was suggested to contribute to tumor progression [11].

The aim of this study was to analyze the levels of IGF-1 and IGF-2 and their transporter proteins IGFBP-1 and IGFBP-2 in the liver of rats with DM, Guerin's carcinoma, and their combination.

MATERIALS AND METHODS

The study included white outbred rats of both sexes weighing 180–220 g obtained from the Research Center for Biomedical Technologies of FMBA, Andreevka branch, the Moscow region. The animals were kept under natural light conditions with free access to water and food. The animals were used in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC), the International Recommendations for Biomedical Research

Involving Animals, and the order of the Ministry of Health of Russia No. 267 "On the Approval of the Rules of Laboratory Practice", dated June 19, 2003. The study was approved by the Ethics Committee at the National Medical Research Center of Oncology (Protocol No. 21/99 of 01.09. 2020).

Animals of each sex were divided into 4 groups of 8 rats each: group 1 – intact animals, group 2 – animals with experimental diabetes, group 3 – animals with subcutaneously inoculated Guerin's carcinoma (control group), group 4 – animals with experimental diabetes and subcutaneously inoculated Guerin's carcinoma growing in presence of alloxan-induced diabetes (treatment group). Experimental diabetes was reproduced by an intraperitoneal alloxan injection at the dose of 150 mg / kg of body weight. Blood levels of glucose were monitored for one week. High blood glucose in the range of 15–30 nmol / l indicated the development of DM.

The rats from the control group and the animals from the main group after 1 week of persistent hyperglycemia received subcutaneous injections of 0.5 ml suspension of Guerin's tumor cells diluted in saline 1:5. The procedure was performed as follows: the assistant shaved off the animal's hair and treated the skin with an iodine alcohol solution 5% downward from the corner of the right shoulder blade; then, using all aseptic techniques described above, the assistant fixed the rat with its back up. The experimenter grasped the treated skin fold with a sterile gloved hand, pierced the skin with a syringe needle and injected the tumor cell suspension. Then the needle was removed, and the injection site was pressed tightly for 1 minute with a cotton swab dipped in 70% alcohol with a small addition of iodine in order to exclude the outflow of the injected suspension. Subcutaneous tumor growth could be recorded three days after the injections of the tumor cell suspension.

At the time of the Guerin's carcinoma inoculation, the average blood glucose values in the animals of the main group (n = 8) were 25.4 ± 1.2 mmol / l, while in the control group of intact animals (n = 8), the values were 5.2 ± 0.3 mmol / l.

10 days after, the animals were decapitated using a guillotine. The levels of IGF-1, IGF-2, IGFBP-1, and IGFBP-2 (Cusabio, China) were measured in the liver of all animals by ELISA using the Infinity F50 TECAN analyzer (Austria). The results were statistically processed using the Statistica 6.0 software. The results were checked for normality of distribution by the Shapiro – Wilk test. The significance of differences between the samples was assessed using the Student's t-test and the Mann – Whitney test. The data were presented as the mean and the standard error of the mean $M \pm m$. The differences were considered significant at $p < 0.05$.

RESULTS

The blood levels of glucose in the intact animals did not have significant sex differences and were on average 5.4 ± 0.5 mmol / l. In the rats with inoculated Guerin's carcinoma, the blood glucose levels were on average 5.1 ± 0.43 mmol / l. In the animals with alloxan-induced T1DM, the blood glucose averaged 22.5 ± 2.1 mmol / l; in the rats of the main group, the blood glucose levels were 25.3 ± 2.4 mmol / l at the time of tumor onset.

The levels of IGF-1 and IGF-2 in the liver of male outbred white rats were 1.4 times lower than in females, and the levels of IGFBP-1 in male rats were 1.8 times lower than in female ones ($p < 0.05$) (Table 1).

Table 1

Levels of insulin-like growth factors and transporter proteins in the rat liver, ng / g of tissue, $M \pm m$

Groups	IGF-1	IGFBP-1	IGF-2	IGFBP-2
Females				
Intact	1166.5 ± 98.7[4]	123.2 ± 11.4[4]	14.6 ± 1.2[4]	230.1 ± 21.4
Диабет	1480.4 ± 120.1[1, 3]	200.5 ± 17.6[1, 3, 4]	32.9 ± 2.9[1, 3]	454.0 ± 43.6[1, 3, 4]
Guerin's carcinoma	903.5 ± 87.4[2, 4]	77.4 ± 6.7[1, 2, 4]	16.0 ± 1.4[2]	197.4 ± 18.5[2, 4]
DM + carcinoma	1322.2 ± 113.5	140.1 ± 12.0[2, 3]	39.8 ± 3.5[1, 3]	425.7 ± 39.2[1, 3, 4]
Males				
Intact	843.6 ± 81.3	66.6 ± 5.8	10.8 ± 0.9	202.9 ± 19.1
DM	1269.2 ± 97.8[1]	140.5 ± 10.3[1, 3]	51.0 ± 4.3[1, 3]	704.8 ± 65.7[1, 3]
Guerin's carcinoma	1233.7 ± 101.6[1]	93.8 ± 8.3[1, 2]	18.7 ± 1.6[1, 2]	298.2 ± 21.4[1, 2]
DM + carcinoma	1526.3 ± 132.5[1]	152.1 ± 11.4[1, 3]	43.0 ± 3.7[1, 3]	726.7 ± 64.3[1, 3]

Note: [1] statistically significant differences compared with intact animals; [2] statistically significant differences compared with animals with DM; [3] statistically significant differences compared with animals with carcinoma; [4] statistically significant differences compared with males of the corresponding groups, $p < 0.05$ (here and in Table 2).

Female rats with DM showed an increase in the levels of IGF-1 and IGF-2 in the liver samples by 1.3 and 2.3 times, respectively ($p < 0.05$), and a rise in IGFBP-1 and IGFBP-2 by 1.6 times and by 2 times, respectively, compared with the values in the intact animals. The growth of inoculated Guerin's carcinoma downregulated only the levels of IGF-1 and IGFBP-1 in the liver by 1.3 and 1.6 times, respectively ($p < 0.05$), and did not affect the levels of IGF-2 and IGFBP-2. As a result of the growth of Guerin's carcinoma in the presence of DM, the levels of IGF-1 in the liver did not differ from those in the animals with DM and were 1.5 times higher ($p < 0.05$) than in the animals with malignant tumors only, while the levels of IGF-2 were 2.5 times higher than the values in the animals with Guerin's carcinoma.

As for IGF-binding proteins, the levels of IGFBP-1 and IGFBP-2 in the liver of the animals from the treatment group exceeded the values in the animals with independent tumor growth by 1.8 times and 2.2 times, respectively, while IGFBP was 1.4 times lower than in the rats with DM.

In male rats, DM upregulated the levels of both IGF-1 and IGF-2 by 1.5 times and 4.7 times, respectively ($p < 0.05$), compared with the values in the intact animals, as well as the levels of IGFBP-1 and IGFBP-2 by 2.1 times and 3.5 times, respectively (Table 1).

The growth of Guerin's carcinoma also increased the levels of IGF-1 and IGF-2 by 1.5 and 1.7 times, respectively ($p < 0.05$), and the levels of IGFBP-1 and IGFBP-2 by 1.4 and 1.5 times, respectively ($p < 0.05$), compared with the values in the intact animals. The studied parameters were elevated in the animals with malignant tumors or DM alone; the combined growth of Guerin's carcinoma in the presence of DM demonstrated quite similar changes, compared with independent processes.

Changes in the IGF / IGFBP ratios were found in all the groups of animals (Table 2) despite the fact that elevated levels of IGF-1, IGF-2, and IGF-binding proteins were determined in the liver of both male and female animals with independent and combined pathologies, except for the growth of Guerin's carcinoma in females. Thus, in females with DM, all ratios decreased: IGF-1 / IGF-2 – by 1.8 times ($p < 0.05$), IGF-1 / IGFBP-1 – by 1.8 times ($p < 0.05$), IGF-1 / IGFBP-2 – by 1.5 times ($p < 0.05$), IGF-2 / IGFBP-1 – by 1.4 times ($p < 0.05$), and IGF-2 / IGFBP-2 – by 1.3 times ($p < 0.05$) (Table 2).

Females with growing Guerin's carcinoma showed a decrease in the IGF-1 / IGFBP-1 ratio in the liver by 1.4 times ($p < 0.05$), IGF-2 / IGFBP-1 – by 1.8 times ($p < 0.05$), and IGF-2 / IGFBP-2 – by 1.3 times ($p < 0.05$). In combined pathologies, the ratios of IGF-1 / IGFBP-1 and IGF-2 / IGFBP-1 decreased by 1.4 and 1.7 times, respectively ($p < 0.05$), compared with DM, and by 1.7 and 1.4 times, respectively ($p < 0.05$), compared with independent growth of Guerin's carcinoma.

Table 2

Ratios of insulin-like growth factors and transporter proteins in the liver of rats, $M \pm m$

Groups	IGF-1/IGFBP-1	IGF-1/IGFBP-2	IGF-2/IGFBP-1	IGF-2/IGFBP-2
Females				
Intact	79.9 ± 7.1	5.1 ± 0.45	8.4 ± 0.76	0.5 ± 0.04
DM	45.0 ± 4.2[1, 4]	3.3 ± 0.27[1, 3, 4]	6.1 ± 0.52[1, 3, 4]	0.4 ± 0.03[1, 4]
Guerin's carcinoma	56.5 ± 5.5[1]	4.6 ± 0.42[2]	4.8 ± 0.33[1, 2]	0.4 ± 0.03[1, 4]
DM + carcinoma	33.2 ± 2.9[1, 2, 3]	3.1 ± 0.29[1, 3]	3.5 ± 0.31[1, 2]	0.3 ± 0.02[1, 2, 3, 4]
Males				
Intact	78.1 ± 6.7	4.2 ± 0.38	6.2 ± 0.54	0.3 ± 0.02
DM	24.9 ± 2.3[1, 3]	1.8 ± 0.15	2.8 ± 0.27[1, 3]	0.2 ± 0.019[1, 3]
Guerin's carcinoma	66.0 ± 5.8[2]	4.1 ± 0.34	5.0 ± 0.43[1, 2]	0.3 ± 0.02[2]
DM + carcinoma	35.5 ± 3.1[1, 2, 3]	2.1 ± 0.19	3.5 ± 0.32[1, 2, 3]	0.2 ± 0.018[1, 3]

The IGF-1 / IGFBP-2 ratio decreased by 1.5 times ($p < 0.05$) compared with animals with Guerin's carcinoma, and the IGF-2 / IGFBP-2 ratio decreased by 1.3 times ($p < 0.05$) compared with the animals with solitary pathologies.

In males, the ratio of IGF to IGF-binding proteins decreased by 1.5–3.1 times ($p < 0.05$) in the DM group, but not in the group with independent tumor growth, compared with the intact animals (Table 2). The IGF / IGFBP ratio in the treatment group was 1.5–2 times lower ($p < 0.05$) than in the group with independent tumor growth. Compared with the group of males with DM, the ratios in the treatment group did not differ significantly, except for IGF-1 / IGFBP-1, which was 1.4 times higher.

DISCUSSION

The levels of IGFs and IGFBP-1 in the liver of outbred white rats were sex-specific. The levels of IGF-1, IGF-2, and IGFBP-1 in the liver of males were lower than in females. Changes in the sex specificity of IGF and IGF-binding proteins were revealed in the liver of rats with DM: the levels of IGF-1 in males did not differ from those in females, and the levels of IGF-2 and IGFBP-2 exceeded those in the liver of females. The differences in the content of IGF and IGFBP in healthy and pathological tissues of the liver in females and males are associated with the main sex steroids – estrogens and androgens. Studies have shown that biologically available estrogen and testosterone are elevated in women with DM [12], while the total testosterone concentration in men with DM is lower than in men without DM [13]. Although the mechanism remains unclear, it may be explained by different affinities of steroids to sex hormone-binding globulin (SHBG) [14, 15]. SHBG synthesis declines with an increase in the blood levels of glucose and insulin, which elevates the levels of free estrogen and testosterone. This is the main reason why DM may play an important role in protecting men from prostate cancer, but not protecting women from breast and endometrial cancers. High levels of free estrogens and androgens are associated with a higher risk of developing many types of cancer, such as breast, endometrial, and prostate cancers [16]. The subcutaneous transplantation of Guerin's carcinoma leveled the initially existing sex specificity of IGF and IGFBP-1 in the liver, and the levels of IGF-1 and IGFBP-2 in males exceeded those in females, while the values of IGF-2 and IGFBP-1 did not differ from those in females.

IGFs and proteins that affect their bioavailability act in an autocrine / paracrine manner, reducing inflammation and fibrosis in the liver and inhibiting the activation of hepatic stellate cells [17]. High insulin levels stimulate liver cells to produce IGF, and IGF bind to IGF receptors (IGF-R) to activate some metabolic and mitogenic signaling pathways that regulate proliferation, differentiation, and apoptosis of cancer cells [7]. Our study established that IGF levels in the liver increased in both male and female rats with DM, but only in males with Guerin's carcinoma. It is worth noting that IGFs bind to IGFBP, including IGFBP-2 [18]. Our study revealed an increase in IGFBP levels in the liver of animals of both sexes with DM. Elevated levels of IGFBP-1 and IGFBP-2, in particular in older men, were suggested to be associated with a decline in insulin sensitivity [8].

The significant increase in IGFBP-2 in the liver of rats under the influence of DM, both alone and in a combination with developing malignant process, was worth noting. The role of IGFBP-2 in physiological and pathological conditions is still not fully understood. However, IGFBP-2 has been related to metabolic syndrome, T2DM, and fatty liver disease. Altered IGFBP-2 secretion may indicate cellular dysfunction of hepatocytes [19]. IGFBP-2 is believed to be a non-invasive biomarker of lipid accumulation in the liver indicating the disease progression [1].

Changes in the levels of IGF and IGFBP during the growth of Guerin's carcinoma in the presence of DM are unidirectional in animals of both sexes and characterized by increasing levels of the studied substances compared with the values in the intact animals. DM as an endocrine disease induced in experimental animals determines the status of the IGF-1 axis. This fact was confirmed in female rats, as the growth of Guerin's carcinoma alone caused a decrease in IGF-1 in the liver, while in the presence of DM, its levels, as well as the levels of IGFBP, increased.

Thus, Guerin's carcinoma and DM cause multidirectional changes in IGF and IGFBP in the liver of females, but unidirectional changes in males. The development of Guerin's carcinoma in the presence of DM reduces sex differences in the studied parameters, and DM changes the metabolic profile of the liver in animals of both sexes. Only experimental studies can solve a number of issues related to the pathogenesis of cancer in presence of comorbid diseases [20].

The global incidence of DM and cancer is believed to be growing rapidly due to changes in lifestyle and increasing life expectancy. Since the internal heterogeneity of DM and cancer complicates research, many questions remain, and the main ones are how endocrine comorbidity affects the risk, course and outcome of malignant disease and what the main biological mechanisms of the malignant development in the presence of such a serious concomitant pathology as DM are. Further research is required to provide a broader range of preventive and therapeutic options for treating cancer patients with DM.

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Frantsiyants E.M., Kotieva I.M., Kaplieva I.V. – conception and design of the experiment. Surikova E.I., Neskubina I.V., Pogorelova Yu.A. – analysis and interpretation of the results. Frantsiyants E.M., Bandovkina V.A., Shaposhnikov A.V. – preparation and editing of the manuscript, critical revision of the manuscript for important intellectual content. Trepitaki L.K., Morozova M.I., Nemashkalova L.A., Sheiko E.A. – carrying out of the experiment. Pogorelova Yu.A., Cheryarina N.D. – carrying out of the ELISA analysis. Frantsiyants E.M., Bandovkina V.A., Kaplieva I.V. – final approval of the manuscript for publication.

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Screening of local hyaluronic acid injection modes to increase the efficiency of treating crush injury of soft tissues

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ABSTRACT

Aim. To study the state of microcirculation and metabolic activity of the soft tissues in the compression area in experimental crush injury after local hyaluronic acid injection and to determine the effective mode of its application.

Materials and methods. The experiments were carried out on 178 male Wistar rats aged 4–4.5 months and weighing 280–340 g. The study design included anesthesia, modeling of crush injury (CI), local injection of 1.75% hyaluronic acid (HA) solution into the compression area, systemic intravenous injection of 0.9% sodium chloride solution daily for 3 days, and a study of microcirculation and metabolism of the soft tissues in the damaged area 3, 7, 14, and 28 days after the injury.

Results. Early (3 hours after the injury) local application of HA for CI improved microcirculation, increased oxygen consumption, and activated oxidative metabolism in the skeletal muscles, which helped reduce the severity of destructive processes in the damaged area. The most effective injection mode was two-fold administration of HA: 3 hours after the compression cessation and additionally 24 hours after the injury.

Conclusion. In the crush injury, early local intramuscular injection of HA into the damaged area in the first few hours after the cessation of compression is a sanogenetically substantiated method for correcting traumatic ischemia of the muscles.

Keywords: crush injury, traumatic ischemia of the muscles, microcirculation, metabolic state, hyaluronic acid, laser Doppler flowmetry

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Скрининг режимов локального применения гиалуроновой кислоты для повышения эффективности лечения компрессионной травмы мягких тканей

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РЕЗЮМЕ

Цель. Изучить состояние микроциркуляции и метаболической активности в мягких тканях области компрессии при экспериментальной компрессионной травме после локального введения гиалуроновой кислоты и определить эффективный режим ее применения.

Материалы и методы. Эксперименты выполнены на 178 самцах крыс линии Вистар весом 280–340 г в возрасте 4–4,5 мес. Дизайн исследования включал в себя обезболивание, моделирование компрессионной травмы (КТ), локальное введение 1,75%-го раствора гиалуроновой кислоты (ГК) в область компрессии, системное внутривенное введение 0,9%-го раствора натрия хлорида ежедневно 3 сут, исследование микроциркуляции и метаболизма мягких тканей области повреждения через 3, 7, 14 и 28 сут после травмы.

Результаты. Раннее (через 3 ч после травмы) локальное применение ГК при КТ улучшает микроциркуляцию, повышает потребление кислорода, активирует окислительный метаболизм скелетных мышц, что способствует уменьшению выраженности деструктивных процессов в области повреждения. Наиболее эффективным является двукратное введение ГК через 3 ч после прекращения компрессии и дополнительно через 24 ч после травмы.

Заключение. При компрессионной травме мягких тканей раннее локальное внутримышечное введение гиалуроновой кислоты в область повреждения в первые несколько часов после прекращения компрессии является саногенетически обоснованным способом коррекции ишемических повреждений.

Ключевые слова: компрессионная травма, травматическая ишемия мышц, микроциркуляция, метаболизм, гиалуроновая кислота, лазерная допплеровская флоуметрия

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Crush injury (CI) (or traumatic ischemia of muscles) is a pathological process that develops after prolonged pressure on soft tissues, mainly skeletal muscles of the extremities, leading to the destruction of muscle fibers and formation of extensive defects of muscle tissue with their subsequent replacement with connective tissue [1]. For normal functioning of skeletal muscles under physiological conditions, as well as during their regeneration after injury, the state of local microcirculation, which provides the metabolic demand for oxygen and nutrients for muscle tissue, is of particular importance [2]. High microcirculation in the muscles stimulates proliferation of myoblasts and facilitates migration of other cells to the injured area [3]. The creation of local matrix-mediated conditions for intercellular interactions promotes activation of a cambial reserve of muscle tissue [4].

One of the promising approaches is the use of biodegradable hydrogels based on hyaluronic acid (HA),

which induce proliferation and migration of poorly differentiated cells [5]. The positive regenerative effect of HA during local application is associated with improved microcirculation, activation of metabolism, and increased angiogenesis and reparative myogenesis [6]. Even a single local injection of HA in the early post-injury period contributes to the restoration of microcirculation and activation of metabolism in the skeletal muscles of the injured area [7]. To determine the therapeutic potential of HA and to identify the mechanisms of its effect on skeletal muscle regeneration in CI, studying the effects of HA on microcirculation and tissue metabolism of the injured area after its single and multiple administration at different times of the post-injury period is required. This will make it possible to develop a method for the local application of HA which will help improve the techniques for treating patients with CI.

The aim of the research was to study the state of microcirculation and metabolic activity of the soft tissues in experimental CI of the limb after local injection of HA into the injured area at different times after the injury.

MATERIALS AND METHODS

The experiments were carried out on 178 adult male Wistar rats weighing 310 ± 30 g, obtained from the Rappolovo nursery (Leningrad region, Russian Federation), in the laboratory of the State Scientific Research Testing Institute of Military Medicine. The rats were 4–4.5 months old. Prior to the experiment, all animals were quarantined for 14 days (air temperature 25 ± 2 ° C, free access to food and water). The study was approved by the local Ethics Committee at the State Scientific Research Testing Institute of Military Medicine (Protocol No. 13 of 22.06.2020) and conducted in accordance with the Directive 2010/63/EC, the Declaration of Helsinki, and the "Rules for Conducting Work with the Use of Experimental Animals".

The study design included the following stages: local anesthesia; CI simulation; local injection of HA into the injured area; systemic intravenous administration of 0.9% sodium chloride solution daily for 3 days; study of microcirculation and metabolism in soft tissues of the injured area at day 3, 7, 14, and 28 after the injury.

For local anesthesia before CI simulation, the rats were intramuscularly injected with zoletil (Virbac, France) and xylazine (Pharmamagist Ltd., Hungary) at a dose of 10 mg / kg for each drug. CI modeling was carried out by controlled mechanical compression of the soft tissues of the thigh according to the technique described above [7].

All animals were divided into 6 groups consisting of 28 animals each: 5 main (I–V) groups and a control group. The animals of the main group I (HA-3) were locally injected with a sterile disposable syringe by the fan technique with an aqueous solution of HA in the injured area once 3 hours after the cessation of the compression; in group II (HA-24), HA was injected once after 24 hours; in group III (HA-48), HA was administered once after 48 hours; in group IV (HA-3 + 24), HA was injected twice, after 3 hours and additionally after 24 hours; in group V (HA-3 + 24 + 48), HA was injected three times, after 3 hours and additionally after 24 and 48 hours. The rats of the control group (n = 28) did not receive local treatment. Intact animals (n = 10) were not subjected to compression.

For the study, HA was used in the form of an aqueous solution of Hyalift 3.5 gel (Aestetic Dermal SL, Spain), which was diluted with 0.9% sodium chloride solution in the 1:1 ratio immediately before administration to obtain 1.75% HA solution with the necessary fluidity. HA solution was injected intramuscularly into the compression area of experimental animals using a fan technique in a total volume of 0.5–0.8 ml per animal. In order to prevent dehydration, all animals with CI for 3 days were injected daily into the tail vein with 0.9% sodium chloride solution at a dose of 2.0 ml / kg of body weight.

In the course of dynamic observation of experimental animals, microcirculation and metabolism in the skeletal muscles of the thigh (pelvic) limb region were assessed using the LAKK-M complex (SPE LAZMA, Russian Federation) at days 3, 7, 14, and 28 after the injury. Under anesthesia, a skin incision was made in the compression area, and a measuring sensor of the device was placed on the thigh muscles. The duration of measurement was 10 min, the depth of probing the muscle tissue volume was 1.0–1.5 mm. In the Laser Doppler Flowmetry (LDF) mode of the device, the amplitude of the parameters M and σ (constant and variable components of microcirculation) was measured; the coefficient of variation (K_v, %) was calculated using the formula: $K_v = \sigma / M \times 100\%$. An increase and (or) decrease in the K_v value indicated improvement and (or) deterioration in the state of microcirculation, respectively.

In the Optical Tissue Oximetry (OTO) mode of the device, the parameter of blood oxygen saturation in the microvasculature of the probed biological tissue

(SO_2, %) was measured, and the index of specific oxygen consumption in the tissue (*U*, relative units) was calculated. The metabolic rate in the skeletal muscles in the injured area was measured in the Laser Fluorescence Diagnosis (LFD) mode, and the amplitude of the fluorescence spectra of the reduced form of nicotinamide adenine dinucleotide (N_{AD}, relative units) and the oxidized form of flavin adenine dinucleotide ($_{FAD}$, relative units) was determined. After that, we calculated the oxygen consumption rate (OCR, relative units) according to the formula: OCR = $_{FAD}$ / N_{AD}. The complex state of microcirculation and metabolism of the skeletal muscles was assessed using the parameter of the efficiency of oxygen metabolism (EOM, relative units), calculated as EOM = M × U × OCR. EOM and OCR are the most informative parameters that characterize the relationship between the state of microcirculation and the intensity of metabolism in tissues.

The levels of myoglobin and potassium in the blood serum were determined using the automated enzyme-linked immunosorbent assay (ELISA) and biochemistry analyzer ChemWell 2910 (Awareness Technology, Inc., USA). An increase in the potassium level indicated the destruction of cells following trauma, while an increase in myoglobin indicated the destruction of muscle tissue [1]. The intact rats were used in the experiment to obtain the average static values of the norm.

The data obtained were processed using the Statistica 10.0 software (StatSoft Inc., USA). After testing the hypothesis for normality of distribution using the Kolmogorov – Smirnov test, the median and the upper / lower quartiles *Me* (Q_{25}-Q_{75}) were calculated. When comparing the data, the nonparametric Mann – Whitney U-test was used. The differences between the values were considered statistically significant at $p < 0.05$.

RESULTS

The death of the animals in the main, control, and comparison groups was observed in the first 4 days and averaged 33%. CT scans showed pronounced microcirculation disorders in the injured area. So, the K_v coefficient in all periods of the follow-up was reduced by 35–49% ($p < 0.05$) relative to the intact rats, with minimum values on days 3–7 after injury. Local circulation disturbances in the injured muscles led to a decrease in tissue oxygen consumption, the SO_2 index initially (on days 3–7) increased by 2.7–2.8 times ($p < 0.05$) and then, by day 28, decreased slightly (by 2.2 times at $p < 0.05$) compared with the intact animals, which indicated high levels of oxygen in the blood that was not metabolized by tissues.

Similar oppositely directed changes were noted in the dynamics of the *U* index. Low oxygen consumption by soft tissues led to disruption of oxidative processes in them. The OCR index on day 3 increased by 90.1% ($p < 0.05$) relative to the values in the intact animals and then recovered on days 7–28. Changes in the EOM parameter were recorded, which was at most reduced by 10.7 times by the end of day 7 ($p < 0.05$) compared with healthy animals. It is possible that the increase in the intensity of metabolic processes in the soft tissues of the injured area in the early periods after the cessation of compression (3 days) was due to the activation of anaerobic metabolism using energy resources (glycogen) located in the preserved muscle fibers. Depletion of these reserves led to a decrease in oxidative processes in tissues against the background of low oxygen consumption by cells.

Local injection of HA in the injured area improved tissue perfusion. The most pronounced positive changes in the studied parameters were observed in the animals that were injected with HA, starting from 3 hours after the cessation of compression (HA-3, HA-3/24, HA-3/24/48), while in the groups of animals with two-fold and three-fold injection, the microcirculation parameters were significantly better. So, the K_v index in the HA-3/24 and HA-3/24/48 groups increased by 38.6–60.3% ($p < 0.05$) compared with the control group during all observation periods and by 9.9–11.2% ($p < 0.05$) compared with the HA-3 and HA-24 groups in the early post-compression period (on days 3–7). HA injection in a later period (48 hours) did not lead to an increase in microcirculation in the injured area. It should be noted that tissue perfusion indices in all the experimental groups showed a positive trend during the entire observation period. According to K_v data, by the end of day 28 in the HA-3/24 and HA-3/24/48 groups, microcirculation in the injured area was restored to values of the intact animals.

Improvement of microcirculation in the injured area contributed to an increase in oxygen consumption by tissues. So, according to SO_2 and *U* data, the intensity of oxygen utilization was the highest in the animals from the HA-3, HA-24, HA-3/24, and HA-3/24/48 groups at all periods of observation. In the groups with two-fold and three-fold injection, the SO_2 parameter on days 3 and 28 was lower by 7.6–16.6% ($p < 0.05$), and the *U* parameter was higher by 20.0% ($p < 0.05$) only on day 28 compared with rats from the HA-3 group. The worst values of oxygen consumption

were observed when HA was injected 48 hours after the cessation of compression. Complete restoration of tissue oxygen saturation was not observed in the compression area after the injection of HA.

All animals from the experimental groups showed pronounced changes in the FAD and NAD amplitude ratio. As a result, OCR in the HA-3 and HA-24 groups was elevated on days 14–28, and in the HA-3/24 and HA-3/24/48 groups – starting from day 7 – by 1.8–2.6 times ($p < 0.05$) compared with the control group. Late use of HA (48 hours after the cessation of compression) led to an increase in metabolism in the early stages (3 days) due to the activation of anaerobic processes and had no significant differences with rats from the control group. Changes in microcirculation, oxygen consumption, and metabolism in the tissues of the compression area were reflected in the dynamics of EOM, which had minimum values on day 7 in all the experimental groups.

The maximum EOM values were observed in the HA-3 and HA-24 groups on days 14–28 and in the HA-3/24 and HA-3/24/48 groups – at all periods of the observation. The use of HA after 48 hours led to a slight increase in EOM only by the end of day 28. Two-fold and three-fold injection of HA significantly increased the efficiency of oxygen metabolism in the injured area (by 37.3–48.2%, $p < 0.05$) on days 14–28 compared with rats from the group with a single early injection of HA after 3 hours. It should be noted that in the rats from the HA-3/24 and HA-3/24/48 groups, the EOM index was restored to the values of the intact animals by the end of the observation period (day 28). Considering that the EOM index reflects the state of tissue perfusion, oxygen saturation, and metabolism, the presented dynamics indicates a positive and (or) negative effect of HA on tissue metabolism with its early and (or) late injection.

Impaired tissue metabolism in CI leads to the development of necrobiotic processes in soft tissues. The breakdown products of damaged muscles enter the systemic circulation with the development of myoglobinemia and hyperkalemia. Early single and (or) two-fold local injection of HA (after 3 and 24 hours) led to a decrease in the myoglobin level by 19.7–38.7% ($p < 0.05$) on days 3–14 compared with the control group. The use of HA at a later time (after 48 hours) did not cause a decrease in myoglobin in the blood. By the end of the observation period, the severity of myoglobinemia decreased, but it remained 4.1 times higher ($p < 0.05$) than in the intact animals. Similar changes were observed in the dynamics of the blood potassium level, which on day 3 decreased on average by 36.8% ($p < 0.05$) in the HA-3/24 group compared with the control group. Later (days 7–28), the concentration of potassium in the blood normalized and was equal to the values in the intact rats.

In Tables 1 and 2, the groups of animals with the best values for microcirculation, oxygen consumption, and oxidative metabolism and with low levels of myoglobin and potassium in the blood serum are at the top of the table; the groups with the worst values are at the bottom.

Table 1

Parameters of microcirculation and metabolism in the soft tissues of the thigh of rats after local injection of hyaluronic acid in different modes in experimental crush injury, *Me* (Q_{25}–Q_{75})

Experimental groups	Observation period after injury, days	*n*	K_v, %	SO_2, %	*U*, rel. units	OCR, rel. units	EOM, rel. units
Intact animals (*n=10*)		10	13.5 (12.5–14.3)	31.6 (30.3–32.9)	3.10 (3.04–3.16)	0.51 (0.43–0.56)	22.5 (21.5–23.6)
Main group IV (HA-3/24), *n = 28*	3	8	10.91^{1-4} (10.4–11.7)	64.1^{1-4} (62.7–65.4)	1.50^{1-3} (1.42–1.66)	0.90^{1} (0.84–0.96)	9.7^{1-3} (8.6–10.9)
	7	8	11.7^{1-4} (11.1–12.3)	78.6^{1-3} (77.5–80.2)	1.22^{1} (1.10–1.30)	$0.63^{2,3}$ (0.57–0.75)	6.1^{1-3} (4.8–7.6)
	14	6	$11.8^{2,3}$ (11.3–12.5)	59.8^{1-3} (58.0–60.6)	1.64^{1-3} (1.59–1.74)	0.79^{1-3} (0.70–0.89)	12.6^{1-4} (11.7–13.8)
	28	6	$12.2^{2,3}$ (11.6–12.9)	39.8^{1-4} (39.0–40.6)	2.46^{1-4} (2.42–2.59)	0.90^{1-3} (0.81–1.00)	22.8^{2-4} (21.6–24.6)
Main group V (HA-3/24/48), *n = 28*	3	8	11.4^{1-4} (10.3–11.8)	63.5^{1-4} (62.1–64.8)	1.54^{1-3} (1.46–1.70)	0.89^{1} (0.85–0.97)	10.1^{1-3} (9.0–11.2)
	7	8	11.8^{1-4} (11.2–12.4)	77.8^{1-3} (76.7–79.4)	1.26^{1} (1.16–1.39)	0.60^{2} (0.52–0.73)	5.8^{1-3} (4.6–7.0)
	14	6	$12.0^{2,3}$ (11.4–12.6)	60.1^{1-3} (59.0–62.1)	1.63^{1-3} (1.58–1.73)	0.81^{1-3} (0.72–0.91)	12.5^{1-4} (11.1–13.8)
	28	6	$12.1^{2,3}$ (11.4–12.7)	40.2^{1-4} (39.4–41.0)	2.44^{1-4} (2.40–2.57)	0.89^{1-3} (0.79–0.94)	21.9^{2-4} (21.3–23.6)

Table 1 (continued)

Experimental groups	Observation period after injury, days	*n*	K_v, %	SO_2, %	*U*, rel. units	OCR, rel. units	EOM, rel. units
Main group I (HA-3), *n = 28*	3	8	9.8[1, 2] (8.7–10.2)	69.4[1-3] (67.5–71.4)	1.38[1, 2] (1.34–1.48)	0.90[1] (0.84–0.98)	8.6[1, 2] (7.5–9.8)
	7	8	10.1[1-3] (9.2–10.5)	79.7[1-3] (77.3–80.9)	1.20[1] (1.10–1.33)	0.50 (0.41–0.58)	4.1[1] (2.7–5.1)
	14	6	10.6[1, 2] (10.0–11.3)	62.1[1-3] (61.0–64.1)	1.58[1-3] (1.55–1.65)	0.77[1-3] (0.70–0.82)	8.5[1-3] (7.5–10.1)
	28	6	11.1[1, 2] (10.4–11.7)	47.7[1-3] (46.8–48.6)	2.05[1-3] (2.02–2.13)	0.89[1-3] (0.79–1.00)	16.6[1-3] (15.2–17.8)
Main group II (HA-24), *n = 28*	3	8	9.3[1, 2] (8.6–10.3)	72.8[1-3] (70.9–73.9)	1.35[1] (1.17–1.42)	0.93[1] (0.85–0.98)	8.4[1, 2] (7.2–9.6)
	7	8	9.7[1-3] (9.0–10.7)	82.5[1-3] (80.2–83.8)	1.19[1] (1.03–1.33)	0.46 (0.34–0.56)	3.4[1] (2.0–4.4)
	14	6	10.4[1, 2] (9.5–11.2)	65.6[1-3] (64.2–66.5)	1.49[1-3] (1.46–1.56)	0.67[1, 2] (0.57–0.77)	6.7[1, 2] (5.2–7.8)
	28	6	11.0[1, 2] (10.3–11.6)	45.4[1-3] (44.0–46.7)	2.16[1-3] (2.08–2.17)	0.79[1-3] (0.70–0.88)	15.2[1-3] (13.9–16.5)
Main group III (HA-48), *n = 28*	3	8	8.3[1] (7.7–8.8)	80.1[1, 2] (77.7–81.5)	1.22[1] (1.18–1.32)	0.95[1] (0.89–1.03)	6.4[1] (5.2–7.5)
	7	8	7.9[1] (7.2–8.7)	86.1[1] (84.4–88.3)	1.14[1] (1.02–1.26)	0.41 (0.34–0.49)	2.8[1] (1.6–4.3)
	14	6	9.2[1] (8.3–9.9)	74.5[1, 2] (72.7–78.0)	1.32[1] (1.24–1.39)	0.53 (0.44–0.62)	4.5[1] (3.4–6.1)
	28	6	9.9[1] (9.1–10.4)	57.1[1, 2] (55.3–58.0)	1.72[1, 2] (1.71–1.74)	0.57 (0.48–0.65)	8.4[1, 2] (7.2–9.7)
Control group (without local injection), *n = 28*	3	8	7.4[1] (6.7–8.0)	86.5[1] (84.6–87.6)	1.11[1] (0.95–1.15)	0.97[1] (0.89–1.06)	6.0[1] (5.1–6.9)
	7	8	6.8[1] (6.3–7.8)	89.7[1] (87.4–91.0)	1.07[1] (0.91–1.21)	0.36 (0.27–0.44)	2.1[1] (0.8–3.2)
	14	6	8.5[1] (7.7–9.2)	81.4[1] (80.0–82.3)	1.20[1] (1.09–1.23)	0.35 (0.26–0.45)	2.6[1] (1.1–3.7)
	28	6	8.8[1] (7.9–9.5)	69.1[1] (67.4–70.5)	1.42[1] (1.39–1.47)	0.34 (0.28–0.48)	3.7[1] (2.3–4.9)

[1, 2, 3, 4] $p < 0.05$ – differences with parameters in animals of intact, control, HA-48, and HA-3 groups (here and in Table 2).

Table 2

Dynamics of myoglobin and potassium levels in the blood serum of rats after local injection of hyaluronic acid in different modes with experimental crush injury *Me* (Q_{25}-Q_{75})

Experimental groups	Observation period after injury, days	*n*	Myoglobin, ng / ml	Potassium, mmol / l
Intact animals *n=10*		10	77.5 (69.0–90.0)	4.1 (3.9–4.4)
Main group II (HA-3/24), *n* = 28	3	8	780.4[1, 2] (731.8–829.0)	4.8[1, 2] (4.5–5.4)
	7	8	647.5[1-3] (583.0–660.7)	4.6 (4.2–4.9)
	14	6	409.0[1, 2] (357.1–461.1)	3.6 (3.1–4.0)
	28	6	322.4[1] (272.2–374.4)	3.6 (3.3–4.4)
Main group I (HA-3/24/48), *n* = 28	3	8	786.2[1, 2, 3] (740.9–831.2)	4.9[1, 2] (4.7–5.3)
	7	8	634.2[1, 2] (586.8–679.1)	4.5 (4.0–4.8)
	14	6	411.4[1, 2] (368.9–453.0)	3.6 (3.3–3.8)
	28	6	318.1[1] (267.3–370.3)	3.6 (3.3–4.2)
Main group III (HA-3), *n* = 28	3	8	776.1[1, 2] (716.9–838.3)	5.0[1, 2] (4.7–5.6)
	7	8	694.0[1, 2, 3] (630.4–707.3)	4.1 (3.5–4.4)
	14	6	447.2[1, 2] (386.0–507.2)	3.5 (3.3–3.9)
	28	6	354.6[1] (303.9–406.4)	4.4 (4.1–5.0)
Main group IV (HA-24) *n* = 28	3	8	794.9[1, 2] (741.2–848.4)	5.8[1, 2] (5.5–6.4)
	7	8	768.2[1, 2] (721.2–815.7)	4.5 (3.9–4.8)
	14	6	549.3[1, 2] (497.9–601.6)	3.6 (3.3–3.8)
	28	6	346.5[1] (295.7–398.3)	3.8 (3.5–4.4)
Main group V (HA-48), *n* = 28	3	8	912.6[1] (861.9–964.7)	5.8[1, 2] (5.5–6.4)
	7	8	804.1[1] (757.1–851.6)	4.5 (3.9–4.8)
	14	6	584.5[1] (542.0–626.1)	3.4 (3.2–3.8)
	28	6	340.0[1] (289.2–392.2)	3.8 (3.5–4.4)

Table 2 (continued)

Experimental groups	Observation period after injury, days	*n*	Myoglobin, ng / ml	Potassium, mmol / l
Control group (without local injection), *n* = 28	3	8	971.0[1] (959.5–1,000.5)	7.6[1] (7.3–8.2)
	7	8	890.5[1] (832.50–960.0)	4.8 (4.2–5.1)
	14	6	701.0[1] (636.0–758.0)	3.6 (3.1–4.0)
	28	6	363.5[1] (318.0–409.0)	3.1 (2.8–3.9)

Thus, local administration of HA early after the cessation of compression (3 hours) in CI led to improvement in microcirculation in the injured area, increase in oxygen consumption, and activation of metabolic processes in tissues, which reduced the severity of destructive processes in them. The best effects were observed in the group of animals, which were injected with HA in the area of compression twice (3 and 24 hours after the cessation of compression).

DISCUSSION

The analysis of the obtained results showed that pronounced microcirculation and metabolism disorders with the predominance of the anaerobic oxidation pathway developed in the soft tissues of the compression area in CI. In response to local injection of HA, improvement in microcirculation in the damaged soft tissues was observed, as evidenced by the increase in the K_v coefficient by 32.4–72.1% ($p < 0.05$) compared with the control group. At the same time, circulation in the injured area had the highest values after local injection of HA after 3 and 24 hours in the early stages (3–7 days) after the cessation of compression.

The restoration of impaired microcirculation contributed to an increase in tissue oxygen saturation, which was reflected in the dynamics of SO_2 and *U* parameters. Thus, after local application of HA, regardless of the dosage regimen, a decrease in SO_2 was observed at all periods of the observation, which indicated an increase in tissue oxygen consumption against the background of circulation restoration. The greatest decrease in SO_2 values was observed in the animals that were injected with HA after 3 and 24 hours. Significant changes were revealed when assessing the *U* index, which tended to be higher when there was an increase in tissue oxygen consumption.

Local injection of HA led to an increase in *U* index mainly at a later time (days 14–28), which may be due to the fact that oxygen was required for intensive repair of soft tissues. Early single or two-fold (after 3 hours and additionally 24 hours) injection of HA contributed to an increase in tissue oxygen consumption not only on days 14–28, but also by day 3 of the observation. The injection of HA at a later time (24 or 48 h) after the injury led to an increase in tissue oxygen uptake only by the end of the observation period (day 28).

The average values of OCR and EOM after local injection of HA, reflecting the intensity of oxidative metabolism in compressed soft tissues corresponded to the microcirculatory status and oxygen consumption parameters. The most significant positive effect of HA was observed in the groups with its early administration (3 and 24 hours after the cessation of compression): in the HA-3 and HA-24 groups on days 14–28, in the HA-3/24 group – on days 7–28. Late injection of HA (after 48 hours) did not significantly affect the intensity of metabolic processes in the tissues of the injured area. The dynamics of EOM had a clear dependence on the mode of HA injection. Thus, local injection of HA in the HA-48 group contributed to an increase in its values by day 28, in the HA-24 group – by days14–28, and in the HA-3, HA3/24, and HA-3/24/48 groups – throughout the observation period compared with the values in control group.

In case of soft tissue damage, therapeutic measures are primarily aimed at replacing the tissue defect and activating regeneration mechanisms, which are possible only with the restoration of intercellular interactions [8]. HA in the injured area, possessing hydrophilicity and high biocompatibility, provides conditions for diffusion of nutrients and oxygen and migration of cells of the immune system [9].

The positive effects of HA in the early post-injury period are associated with facilitating the migration of immune cells capable of limiting the necrotic zone and disposing of destroyed cellular structures from tissues. The study showed that the most pronounced effect of HA was manifested with its early local administration (after 3 hours), while late injection (after 48 hours) was ineffective. This can be explained by the physiological mechanisms of restoration of injured tissues, which are activated immediately after the injury. These processes are genetically determined and proceed under the control of neurohumoral mechanisms [10].

In the early periods after the termination of compression in CI, local defense systems are activated in the tissues of the injured area in response to massive se-

cretion of inflammatory mediators, which is necessary to localize the lesion, eliminate the factors that caused it, and remove decay products. All this contributes to the preservation of the metabolic activity of cells in necrobiosis and triggers regeneration. Therefore, the biological action of HA is implemented at both cellular and intercellular levels. The cellular effects of HA are due to its ability to activate the receptors of cell membranes and change ion fluxes; intercellular effects are aimed at maintaining tissue homeostasis by forming a biochemically stable intercellular environment. In this regard, it is obvious that local injection of HA in the injured area in CI is ineffective when it is carried out later (48 hours after the injury). At the same time, single (after 3 hours) and multiple early (after 3 hours and 24 hours) local injection of HA is pathogenetically substantiated and has a positive effect on the formation of sanogenesis in CI.

CONCLUSION

1. Early local injection of HA in the injured area 3 hours after the cessation of compression improves microcirculation, increases oxygen consumption by tissues, positively affects the metabolism in the skeletal muscles, and reduces the severity of destructive processes in them.

2. The most effective method for correcting CI is early repeated injection of HA in the compression area 3 and 24 hours after the injury.

3. In case of CI, a single or multiple intramuscular injection of HA in the injured area in the early post-compression period is a sanogenetically substantiated method for correcting traumatic ischemia of muscle.

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Features of the clinical presentation and course of community-acquired pneumonia against the background of type 2 diabetes mellitus

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ABSTRACT

Community-acquired pneumonia remains the leading infectious cause of death around the world. Many factors influence the prognosis and outcome of this disease. Compared with healthy individuals, patients with diabetes mellitus are at increased risk of respiratory tract infections, such as community-acquired pneumonia. Diabetes mellitus contributes to the development of pulmonary thrombotic microangiopathy, changing the functional state of the lungs.

In numerous studies involving patients with diabetes mellitus, data on the state of the lungs were obtained by instrumental tests, such as spirometry, ventilation / perfusion scintigraphy, perfusion computed tomography, and diffusing capacity of the lungs for carbon monoxide. In patients with community-acquired pneumonia, diabetes mellitus causes vague clinical symptoms, leads to a severe course of the disease, and contributes to development of complications. Diagnosing the functional state of the lungs in patients with community-acquired pneumonia against the background of diabetes mellitus has not been studied.

Keywords: community-acquired pneumonia, diabetes mellitus

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Особенности клиники и течения внебольничной пневмонии на фоне сахарного диабета 2-го типа

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РЕЗЮМЕ

Внебольничная пневмония остается ведущей инфекционной причиной смерти в мире. Многие факторы влияют на прогноз и исход данного заболевания. По сравнению со здоровыми лицами пациенты с сахарным диабетом подвергаются повышенному риску инфекций дыхательных путей, таких как внебольничная

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пневмония. Сахарный диабет способствует развитию микроангиопатии легких, изменяя функциональное состояние данного органа.

В многочисленных исследованиях пациентов с сахарным диабетом получены данные о состоянии легких инструментальными методами, такими как спирография, вентиляционная сцинтиграфия, перфузионная компьютерная томография и метод определения диффузионной способности легких по монооксиду углерода. У пациентов с внебольничной пневмонией сахарный диабет вызывает стертую клиническую симптоматику, приводит к тяжелому течению внебольничной пневмонии и способствует развитию осложнений. Диагностика же функционального состояния легких у пациентов с внебольничной пневмонией на фоне сахарного диабета не изучена.

Ключевые слова: внебольничная пневмония, сахарный диабет

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

Community-acquired pneumonia is one of the key causes of emergency hospitalization in inpatient facilities of internal medicine and a potentially lethal disease [1, 2]. The World Health Organization (WHO) reports that lower respiratory tract infections, including pneumonia, are still the deadliest infectious diseases. In 2015, 3.2 million people died of these diseases in the world. The severity and outcome of community-acquired pneumonia also depend on such comorbidities as chronic obstructive pulmonary disease (COPD), heart failure, cerebrovascular and kidney diseases, metabolic syndrome, and diabetes mellitus (DM) [2–9].

Many scientific publications thoroughly describe combinations of cardiovascular pathologies and DM as mutually exacerbating conditions. Only few studies analyze the course of community-acquired pneumonia against the background of DM. Most existing publications show data on the incidence of community-acquired pneumonia and analyze the risk of a lethal outcome due to community-acquired pneumonia and concurrent DM.

Currently, DM is one of the most common chronic diseases in the world. In 2016, WHO called it one of the most dangerous noncommunicable epidemics of the XXI century along with cardiovascular diseases, COPD, and cancers [10]. The number of DM patients increases at a rate exceeding the forecasts of experts from the International Diabetes Federation. The research findings from NATION, a large epidemiological study conducted in 2013–2015 and aimed at detecting type 2 diabetes mellitus (T2DM) among the Russian population, show that the prevalence of this disease is 5.4% (about 6.5 million people). DM is characterized by macrovascular and microvascular complications. The latter include complications affecting the kidneys, eye retina, and nervous system [12, 13].

Now there is an increasing amount of data confirming that the lungs are one of the target organs for diabetic microangiopathy. An extensive network of pulmonary capillaries is involved in respiratory metabolism through alveoli that form a single membrane. Hyperglycemia causes structural changes in the pulmonary capillary walls, and these changes have a negative impact on the blood-air barrier, lead to microangiopathy in the DM-affected lungs, and change the functional state of the entire organ [14]. It is proved that DM patients are characterized by pulmonary function impairment, regardless of the duration of the disease [15, 16]. W.A. Davis et al. showed that in the group of DM patients without pulmonary diseases and with 4% reduction in the lung function parameters, the all-cause mortality rate increased by 12% [17].

In addition, some studies show that blood glucose-lowering drugs ensure a 10% increase in lung function parameters (forced expiratory volume in

one second (FEV_1), vital capacity (VC)) compared with the baseline values in T2DM patients [18].

A longitudinal, observational study conducted in the USA in 2005 including 1,443 male patients aged 21–88 years analyzed lung function parameters and blood glucose values and detected statistically low FEV_1 and VC in patients several years before the emergence of DM compared with same aged patients without DM [19].

N. Guvener et al., using the gaseous diffusion method, demonstrated decreased alveolar capillary membrane permeability in DM patients compared with the control group ($p = 0.037$) [20]. At the same time, some studies provide exactly opposite data. In the study by K. Ozşahin, the gaseous diffusion method did not show differences in alveolar capillary membrane permeability between DM patients and healthy people, while it was significantly reduced in the group of DM patients ($p = 0.01$) when ventilation / perfusion scintigraphy was performed [21]. These findings confirm that impaired pulmonary function in DM patients causes subclinical changes in the lung structure that may aggravate such an acute infectious process as community-acquired pneumonia and promote complications.

A review studying patients who are susceptible to invasive pneumococcal infections shows that the risk of such infections, including community-acquired pneumonia, increases in case of DM [22, 23]. Most authors believe that DM contributes to an increase in hospital stay and acts as a predictor of higher mortality in patients with community-acquired pneumonia [24, 25]. For instance, the studies conducted by J.B. Kornum et al. in Denmark in 2007–2008 showed that patients with HbA1c ≥ 9% were statistically often characterized by more severe community-acquired pneumonia and a 60% increase in the risk of hospitalization in relation to pneumonia [26, 27].

The findings of CAPNETZ, a multicenter, prospective cohort study, show that in 2007–2014, the mortality rate within the first month of hospitalization for community-acquired pneumonia amounted to 12.1% in DM patients vs. 3.8% in patients without DM ($p = 0.001$) [28]. The study carried out in Japan in 2005–2011 revealed that the mortality rate within the first month among DM patients hospitalized for community-acquired pneumonia was significantly associated with the degree of hyperglycemia at the moment of hospitalization ($p < 0.0001$) [29]. S. Yende et al. demonstrated that hospitalization for community-acquired pneumonia was a significant risk factor for a lethal outcome within the first year after inpatient treatment for DM patients (hazard ratio (HR) = 1.87) [23]. M. Falcone et al. detected significantly higher annual mortality rates in DM patients hospitalized for community-acquired pneumonia vs. patients hospitalized for other reasons (30.3 vs. 16.8%, $p < 0.001$) [9].

The risk factors for mortality in patients with community-acquired pneumonia against the background of T2DM are bacteremia, septic shock, and comorbidities [30, 31]. It is proved that age has a strong impact on mortality rates in patients with community-acquired pneumonia and DM.

The findings from the NHANES III study (USA) and the analysis of 3,770 death certificates of people aged 65+ years allow to conclude that every year of life increases the risk of death due to community-acquired pneumonia by 16%. It was shown that an increased risk of death due to community-acquired pneumonia is typical of not only DM patients (34.1 per 10,000 person – years), but also of patients with impaired glucose tolerance (16.9 per 10,000 person – years) [23, 32].

The findings from a prospective cohort study (6 years) conducted in Finland (2014) showed that DM and postprandial hyperglycemia newly diagnosed in non-diabetic patients with community-acquired pneumonia were associated with a higher risk of late mortality within several years after experienced community-acquired pneumonia. The mortality rate at the end of the follow-up amounted to 54, 37, and 10% in DM patients, non-diabetic patients with diagnosed postprandial hyperglycemia, and patients without DM and postprandial hyperglycemia, respectively ($p < 0.001$). In addition, hyperglycemia has a prognostic value as a severity criterion for patients with severe community-acquired pneumonia [33]. Only single publications show practically similar mortality data. However, in the authors' opinion, the reason for that was that the studied patients were older and experienced a more severe condition at the moment of admission, so DM had an insignificant impact on their mortality rates [12, 34, 35].

At the same time, many authors are sure that higher mortality rates are typical of DM patients after inpatient treatment of community-acquired pneumo-

nia vs. patients hospitalized for noncommunicable pathologies [23, 34]. Few publications describe the clinical aspects of community-acquired pneumonia with concomitant DM, and these findings are contradictory.

The clinical features of community-acquired pneumonia are different in DM patients. Patients with community-acquired pneumonia against the background of DM experience less pronounced clinical symptoms, such as cough, chills, and acute onset of the disease. This may be the reason why patients with community-acquired pneumonia and DM score less on the CURB-65 and PSI / PORT scales at the moment of admission, however, the duration of hospital stay for patients with community-acquired pneumonia with concomitant DM is longer than for patients without DM [34, 36].

Fever, cough, shortness of breath, and tachycardia, i.e. the classical symptoms of community-acquired pneumonia, disappear, become silent or are absent at all in DM patients. These symptoms are often accompanied by radiographically detected multilobar lung infiltrates, pleural effusion, and empyema. M.A.Saibal reported significant differences ($p < 0.001$) following X-ray examinations. DM patients with community-acquired pneumonia are more often characterized by multisegmental pulmonary lesions than patients without DM [37]. Some other studies show no significant difference in the pulmonary infiltration area in community-acquired pneumonia patients with and without DM [25, 36].

Low symptom intensity makes the disease difficult to diagnose. Extrapulmonary manifestations, such as altered mental state, mental block, hypotonia, and tachypnea, are often prevailing or the only symptoms detected in patients with community-acquired pneumonia with DM during a physical examination. DM patients hospitalized for community-acquired pneumonia more often suffer from metabolic disorders and cardiovascular events [12, 37].

The authors of this study have not found literature data on the features of physical examinations in patients with community-acquired pneumonia and concomitant DM. Community-acquired pneumonia often cannot be diagnosed based only on clinical symptoms and laboratory findings, especially in senior patients and patients with comorbidities, such as DM [38–40]. Community-acquired pneumonia is definitely diagnosed only if infiltration signs are detected using diagnostic radiology methods. At the same time, these instrumental procedures allow to obtain information only on structural changes in the lungs [38, 41, 42].

In some cases, in patients with community-acquired pneumonia and comorbidities, including DM, instrumental methods are not always capable of determining the contribution of pneumonia to the severity of patients' condition. To determine the functional state of the lungs, several methods are used, such as inspiratory and expiratory computed tomography and ventilation / perfusion scintigraphy [43, 21].

In 2006, K.Özşahin conducted a study on alveolar capillary membrane permeability in DM patients and healthy individuals using two methods: gaseous diffusion and ventilation / perfusion scintigraphy. The first method did not detect any difference between the groups. Ventilation / perfusion scintigraphy revealed a significant increase in the half-life of a radiopharmaceutical in DM patients vs. the control group ($T_{1/2}$ = 112.7% and $T_{1/2}$ = 84.6%, p = 0.01) [21]. It was demonstrated that the walls of pulmonary arterioles became thicker due to the development of chronic inflammation [44], an increase in collagen and elastin levels, and fibroblast proliferation [45]. Therefore, ventilation / perfusion scintigraphy is a more sensitive method to determine the alveolar capillary membrane permeability.

In the study by K. Kuziemsky et al., carried out in Poland in 2011, perfusion computed tomography detected upward quantitative changes in the blood flow volume, blood filling, and vascular wall permeability parameters in DM patients compared with the control group of healthy individuals (p = 0.01) [46]. The authors have not found any data on the sensitivity and specificity of diagnostic radiology methods in patients with community-acquired pneumonia and T2DM in the available literature.

CONCLUSION

Therefore, DM is characterized by lung damage accompanied by functional disorders in the lungs. Changes in the pulmonary microvasculature are more diffused than in the renal arteries or vessels in the eye. Therefore, they may remain compensated and have no clinical manifestations for a long time. However, there is much evidence of subclinical manifestations of diabetic lung associated with a

high risk of a lethal outcome in community-acquired pneumonia.

For instance, data on the information value of ventilation / perfusion scintigraphy and perfusion computed tomography in DM patients are available, but the capacities of these methods in patients with community-acquired pneumonia and DM have not been completely unveiled yet. Therefore, the sensitivity and specificity of such methods as inspiratory and expiratory computed tomography and ventilation / perfusion scintigraphy in patients with community-acquired pneumonia and T2DM should be further explored.

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Diuretic resistance in patients with chronic heart failure: mechanisms, prevention, and treatment

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ABSTRACT

The authors analyzed the problem of diuretic resistance (DR) in patients with chronic heart failure (CHF). Most of the symptoms and signs of CHF are associated with hypervolemia and vascular congestion in the systemic and pulmonary circulation. The severity of the latter is the main factor which negatively affects the overall assessment of life satisfaction in patients with CHF. Since the patient, even at the incurable stage of CHF, primarily expects a rapid decrease in the severity of manifestations of decompensation from the prescribed therapy, achieving euvolemia is the essence of its short-term objective. Without diuretics, these immediate effects, according to which most CHF patients judge the qualifications of the doctor, are almost impossible to achieve. Unfortunately, apparently, not a single clinician was able to avoid disappointment in the effectiveness of CHF therapy associated with DR in their practice. As a rule, DR reflects the progressive course of CHF and is often associated with a poor prognosis. The review consistently covers the issues of terminology, diagnosis, pathogenesis, and prevention of DR, which aggravates CHF, and discusses measures aimed at restoring sensitivity to diuretics.

Keywords: chronic heart failure, diuretic resistance, terminology, mechanisms, water and salt restriction, sequential nephron blockade, gliflozines, vaptans, inotropes, vasoconstrictors, glucocorticoids, serelaxin, nesiritide, albumin, ultrafiltration

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Резистентность к диуретикам у пациентов с хронической сердечной недостаточностью: механизмы, профилактика и преодоление

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РЕЗЮМЕ

Проанализирована проблема резистентности к диуретикам (РД) у пациентов с хронической сердечной недостаточностью (ХСН). Большинство симптомов и признаков ХСН связаны с гиперволемией и застоем крови в большом и малом кругах кровообращения. Выраженность последних является основным фактором, негативно влияющим на общую оценку удовлетворенностью жизнью пациентов с ХСН. Так как пациент даже в инкурабельной стадии ХСН в первую очередь ожидает от назначенной врачом терапии быстрого снижения выраженности проявлений декомпенсации, достижение эуволемии представляет собой суть ее краткосрочной задачи. Без диуретиков этих немедленных эффектов, по которым большинство пациентов с ХСН судят о квалификации врача, добиться практически невозможно. К сожалению, по-видимому, ни одному клиницисту не удалось избежать в своей практике разочарования в эффективности терапии сердечной недостаточности, связанного с РД. Как правило, РД отражает прогрессирующее течение ХСН и часто ассоциируется с неблагоприятным прогнозом.

В лекции последовательно рассмотрены вопросы терминологии, диагностики, патогенеза и профилактики РД, отягощающей ХСН, а также обсуждается комплекс мероприятий, направленный на восстановление чувствительности к диуретикам.

Ключевые слова: хроническая сердечная недостаточность, резистентность к диуретикам, терминология, механизмы, водно-солевой режим, секвенциальная блокада нефрона, глифлозины, ваптаны, инотропы, вазоконстрикторы, глюкокортикостероиды, серелаксин, несиритид, альбумин, ультрафильтрация

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

Many definitions of chronic heart failure (CHF) include a description of its main manifestations [1]. In particular, the experts of the European Society of Cardiology define clinically pronounced CHF as "a clinical syndrome characterized by typical symptoms (e.g. shortness of breath, swelling of the ankles, and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, rales, and peripheral edema) caused by a structural and / or functional cardiac abnormality..." [2]. Most of the symptoms and signs presented in this definition are associated with hypervolemia and blood stasis in the systemic and pulmonary circulation. The severity of the latter is the main factor that negatively affects the overall assessment of life satisfaction in patients with CHF, including those with comorbidity [3–5].

Despite the fact that apologists for evidence-based medical practice consider an increase in life expectancy as the main aim of pharmacotherapy for CHF, it is equally important to ensure quality-adjusted life years during treatment [1, 6]. This determines the need for the most complete elimination of CHF symptoms, even at the incurable stage of the disease [7]. Since the patient primarily expects a rapid decrease in the severity of CHF manifestations from the therapy prescribed by the doctor, achieving euvolemia is the essence of its short-term objective [8]. Without diuretics, these immediate effects, according to which most CHF patients judge the qualifications of the doctor, are almost impossible to achieve [9]. It is no coincidence that diuretic therapy is sometimes called a cornerstone of treatment of patients with decompensated CHF [7, 10, 11].

Unfortunately, apparently, not a single clinician was able to avoid disappointment in the effectiveness of CHF therapy associated with diuretic resistance (DR) in their practice [7, 12, 13].

DR is a serious clinical issue which often portends a poor prognosis; hence this issue is actively discussed in peer-reviewed scientific journals [14]. Despite the fact that an expert consensus paper of the European, North American, and Russian task force on the use of diuretics in CHF has been published recently [9, 14, 15], there are still disputes about the most optimal strategy in a situation when it is impossible to achieve euvolemia and / or maintain it.

The titles of articles published in peer-reviewed scientific journals testify to the severity of this issue: "Diuretic resistance in acute decompensated heart failure: a challenging clinical conundrum", "The use of diuretics in heart failure with congestion: we can't judge a book by its cover", "The never-ending quest for the appropriate role of ultrafiltration" [16–18].

The aim of this review was to consider modern views on the issue of diuretic resistance that develops in up to 20–35% of patients with chronic heart failure (in acute decompensated heart failure – in 50% of patients) and on the possibility of overcoming it.

TERMINOLOGY AND DIAGNOSTIC CRITERIA

As the Persian poet As-Samarqandi said, if the disease is not defined, it is impossible to treat it. Since correct recognition of any pathology is not conceivable without a clear understanding of the aim its diagnosis, first of all, one should decide on the terminology. The problem consists in the fact that not all materials reflecting expert consensus statements on the use of diuretics in CHF attempt to define the concept of DR. In turn, the definitions of DR and the criteria for its verification may differ in various documents, which prevents establishing the boundaries of the application of this term (Table 1).

The experts of the American College of Cardiology suggest using the term DR to describe insufficient natriuresis, despite adequate diuretic therapy [14]. This non-quantifiable definition is scientifically grounded, but hardly acceptable for point of care testing, since in the vast majority of cases in clinical practice, the doctor evaluates not natriuresis (as well as not the volume of extracellular fluid during transpulmonary thermodilution, hematocrit, intrathoracic impedance, the number of hyperechoic artifacts on lung ultrasound or ventricular filling pressure according to invasive hemodynamic monitoring), but diuresis followed by a change in body weight [1, 19–21].

It is necessary to understand what adequate diuretic therapy implies, since we can talk about mono- or combination therapy with drugs of different groups, its different duration, as well as a wide range of drug dosages [22]. Since loop diuretics form the basis of diuretic therapy in CHF, the term DR in the vast majority of cases is used to denote resistance specifically to drugs that act along the thick ascending limb of the loop of Henle [9].

In addition to diverse ways of diuretic administration, the choice of which depends on the specific clinical situation, optimal therapy with saluretics involves the use of differentiated approaches to its escalation, taking into account the absence of a linear relationship between the change in the drug dose and the diuretic

effect. For example, increasing the dose of furosemide by 20 mg in a patient who previously received 20 mg of the drug per day will give a significantly greater increase in urine output than 20 mg added to therapy at a dose of 220 mg per day [14].

It is clear that the same dose of a loop diuretic can cause a variable diuretic effect in a population of patients with CHF. Conversely, the same volume of urine can be obtained in different patients with the use of saluretics in a wide range of doses. Therefore, when considering DR, the focus should be placed on evaluating the efficacy of a drug in promoting diuresis rather than on the absolute dose of the diuretic or urine levels [23].

Table 1

Examples of criteria for diagnosing diuretic resistance
I. Based on the assessment of natriuresis
Failure to increase sodium excretion by at least 90 mmol within 72 h with 160 g of furosemide given twice a day orally [24]
FES less than 0.2% [25]
Calculated by the formula: [25] 100 × (Scr × Una) / (Sna × Ucr), where Scr is serum creatinine; Una is urine sodium; Sna is serum sodium; Ucr is urine creatinine.
Cumulative 6-hour natriuresis of less than 50 mmol after intravenous administration of 2–4 mg of bumetanide (median – 3 mg) [26]
Daily sodium excretion of less than 100 mmol after intravenous administration of bumetanide at a dose of 2–4 mg (median – 3 mg) [26]
Sodium concentration of less than 50 mEq / l or Na+ / K+ ratio of less than 1.0 in a urine sample obtained 8 hours after diuretic administration [27]
The ratio of the sodium concentration / furosemide concentration in the urine is less than < 2 mmol / mg [28]
Expected cumulative natriuresis of less than 100 mmol 6 hours after intravenous administration of a loop diuretic [26]
Calculated by the formula: GFR×(BSA/1.73)×(Scr/Ucr)×150×(Una/1000); [26] where GFR is the glomerular filtration rate; Scr is serum creatinine; BSA is body surface area; Una is urine sodium; Ucr is urine creatinine
II. Based on the assessment of diuresis
Diuresis of less than 1,400 ml on the first day after the prescription of 40 mg of furosemide (or an equivalent dose of another diuretic) [28]
Diuresis of less than 2,000 ml / day after intravenous administration of 40 mg of furosemide [29]
III. Based on body weight dynamics
No weight loss within 48–96 hours after the initiation of therapy with furosemide 40 mg / day (or an equivalent dose of another diuretic) [28, 30]
IV. Based on the dose and route of administration of diuretics
The need for intravenous administration of furosemide at a dose of more than 80 mg / day [31]
Persistent stagnation despite the use of furosemide at a dose equal to or exceeding 80 mg / day [32]
The need for taking furosemide at a dose greater than 3 mg / kg / day (or an equivalent dose of another loop diuretic) [33]

Note: FES (fractional excretion of sodium) is a part of the electrolyte excreted with urine from the total amount passed through glomerular filtration.

Since impaired sensitivity to diuretics limits the possibility of achieving euvolemia, failure to achieve the so-called dry weight when using high doses of diuretics (primarily loop diuretics) (the ideal weight of the patient without excess body fluid) can be used as a DR criterion in clinical practice [9, 34–36]. Dry weight is the term most often used by renal replacement therapists to describe the patient's weight when they are euvolemic. This is the weight above which symptoms and signs of fluid retention are observed, and below which the patient develops hypotension (with normal dry weight, systemic blood pressure, as a rule, is not lower than 110 / 50 mm Hg) and often signs of kidney disease [37, 38].

The desire of physicians, starting to treat a patient with CHF, to achieve euvolemia, according to the figurative expression of K. Watson et al. [38], is as clear as that "the night is dark and the day is bright". However, the lack of a reliable and at the same time simple point of care method for determining euvolemia (dry weight) leads to the fact that on the way to the coveted euvolemia, internists have to give it a go and be ready to make mistakes, empirically trying to establish the optimal point for discontinuing / de-escalating anti-edematous therapy, determine the time for its intensification without a delay, and timely recognize the development of DR [9, 35].

The situation is deteriorated by the fact that clinicians are forced to act in a vacuum of generally accepted qualitative and quantitative criteria for DR verification. Unfortunately, we have to state that we should not expect quick changes for the better, since identification of a decisive diagnostic rule in this case can be compared with an attempt to solve an equation with many unknowns, which is known to have an infinite number of solutions.

Finally, we should not forget about the very common pseudo-resistance to diuretics, which the doctor

must exclude before speaking about DR. Pseudo-DR should be considered when a patient is not receiving optimal diuretic therapy for any reason. For example, when the doctor chooses an inadequate saluretic therapy strategy (prescribing a low dose of a loop diuretic or an intermittent use of it, as well as an unsuccessful combination with drugs that reduce the effectiveness of the diuretic) or in case of poor patient's adherence to treatment. Moreover, before discussing DR, the doctor should exclude edematous syndrome secondary to venous insufficiency, impaired lymph circulation (lymphedema), hypoalbuminemia, and endocrine gland disorders (for example, hypothyroidism or syndrome of inappropriate antidiuretic hormone secretion), as well as that associated with drug therapy (for example, with the use of dihydropyridine derivatives) [20].

MECHANISMS OF DEVELOPMENT OF DIURETIC RESISTANCE

The pathogenesis of fluid retention in CHF cannot be reduced to a single mechanism, since the expansion of extracellular fluid is a complex multistage process [39]. In turn, the pharmacokinetics and pharmacodynamics of saluretics include a number of discrete stages, and diverse disturbances at each stage provide the key to understanding the heterogeneity of the DR mechanisms [9, 14, 40].

Identification of the mechanism(s) of DR can contribute to the development of an effective individual strategy for improving the response to diuretics in a patient with CHF. It is important to consider that many mechanisms of DR have been described in studies performed in a population of healthy individuals and patients with arterial hypertension or chronic kidney disease. The intuitive conclusion that these results are fully applicable to patients with heart failure may be erroneous [20]. Thus, it is obvious that kidney dysfunction (a decrease in the glomerular filtration rate) as a cause of DR in patients with CHF is less significant than in chronic kidney diseases. We believe that, taking into account a large number of phenotypes of heart failure, which is not accidentally called multifaceted, caution is also necessary when extrapolating the results of studies in a cohort of patients with acute heart failure to the population of patients with CHF.

Z.I. Cox and J.M. Testani in their work "*Loop diuretic resistance in a patient with acute heart failure*" [36] identified the extrarenal and renal forms of DR and systematized the key mechanisms of development of the latter based on identifying predominantly involved segments of the nephron. The adapted results of this systematization, supplemented by other authors, are presented in Table 2.

Table 2

The main mechanisms of development of diuretic resistance [14, 36, 41]

Prerenal disorders	The level of real shifts		
	Before the loop of Henle	The loop of Henle	After the loop of Henle
Cardiorenal syndrome (types 1 and 2) Pathology of renal blood flow Hypoalbuminemia High sodium intake Impaired absorption of the diuretic Increased intra-abdominal pressure	Reduction of the number of nephrons Decreased glomerular filtration rate Competition for the penetration of diuretics into the nephron among organic anions Albuminuria	Low dose of the loop diuretic Non-optimal frequency of loop diuretic prescription Weak natriuretic response at the level of the loop of Henle Hypochloremic alkalosis	Distal tubular hypertrophy Hyperfunction of the distal tubules

Note: the list is not comprehensive.

The enumeration and detailing of the mechanisms of primary and secondary DR could be continued. For example, increased expression of the pendrin gene, polymorphism of other genes encoding ion transporters, cotransporters (symporters) or exchangers (antiporters), as well as vasopressin-induced activation of the incorporation of aquaporin-2 channels into the apical membrane of collecting duct epithelial cells [14, 41, 42]. Their prevalence and clinical and prognostic significance remain unclear and require future study. It is assumed that the most common forms of DR are associated with structural and functional changes that develop at the level of the distal tubules [36], and the most important causes of resistance to diuretics include compensatory sodium reabsorption in the distal tubules (regardless of the fact that only 10% of sodium is normally reabsorbed in this segment of the nephron) and a low dose of a loop diuretic [14]. However, the latter has nothing to do with DR, since true resistance, as noted above, implies the presence of adequate di-

uretic therapy that can provide a sufficient intrarenal diuretic concentration [20].

Despite the concurring opinion of the majority of experts on the association of DR with a poor prognosis in patients with heart failure (especially with acute decompensated heart failure) [41, 43], the mechanisms of resistance to saluretics cannot be considered as solely pathological [20]. The physiological meaning of the mechanisms of renal autoregulation and neurohormonal reactions is to eliminate excessive deviations in the fluid – electrolyte balance that develop after massive natriuresis already at the start of therapy with high doses of diuretics [44].

The first dose of a diuretic often causes encouraging diuresis. However, when the volume of extracellular fluid decreases after profuse diuresis, activation of the sympathetic and renin – angiotensin – aldosterone systems leads to the development of the so-called inhibition phenomenon [20, 44, 45]. The impossibility in all cases to effectively modulate the severity of the inhibition phenomenon with the help of neurohumoral blockers makes it possible to discuss alternative, volume-independent mechanisms of early DR. In particular, a hypothesis has been put forward about the memory effect of the epithelium of the renal tubules on the effect of diuretics [46]. Regardless of its mechanism, inhibition of the effect within certain limits is useful because it saves from diabetes, on the one hand, and, paradoxically, prevents the development of hypovolemia-related DR. If the initial diuretic effect associated with an increase in the excreted fraction of Na^+ by 20% persisted with continuous infusion of a loop diuretic, at a glomerular filtration rate of 120 ml/ m in, the patient would lose 280 grams of salt and 50 liters of osmotically bound water per day [20].

In contrast to early DR associated with the inhibition phenomenon, late refractoriness develops after weeks and months of continuous diuretic therapy. Diuretic-induced chronic intraluminal overload of the distal convoluted tubules and collecting ducts with Na^+ and Cl^- ions triggers structural and functional adaptation of the kidneys [40]. The leading mechanism of late DR is hypertrophy and hyperfunction of cells of the simple cuboidal epithelium of the distal convoluted tubules, as well as primary and intercalary cells of the collecting duct epithelium, which are sometimes erroneously called tubules [20].

Remodeling of the distal segment of the nephron and collecting ducts is associated with activation of the thiazide-sensitive Na^+-Cl^- cotransporter, aldosterone-sensitive epithelial sodium channel, chloride – bicarbonate exchanger (pendrin), which leads to an increase in tubular sodium reabsorption [47]. Thus, with chronic intravenous use of a high dose of the loop diuretic (median is 160 mg of furosemide per day), fractional sodium excretion in patients with acute heart failure increased by only 4.8% [48], indicating that about 70% of sodium ions leaving the loop of Henle undergo distal tubular reabsorption [20].

Along with remodeling and hyperfunction of the distal tubules, the development of hypochloremic metabolic alkalosis, usually caused by the simultaneous use of loop and thiazide diuretics, which promote the retention of bicarbonates, can also be noted as an important mechanism of DR. Even mild metabolic alkalosis, which is the most common acid – base disorder in CHF patients, leads to a decrease in the natriuretic effect of the loop diuretic by about 20% [46].

PREVENTION AND MANAGEMENT OF DIURETIC RESISTANCE

CHF is a syndrome and not a disease, and when developing individual treatment strategy, its etiological heterogeneity should be taken into account [49]. Properly selected treatment of the disease underlying CHF in many cases can significantly reduce the severity of manifestations of cardiac decompensation, and sometimes allows the patient to completely eliminate them (for example, after successful surgical correction of heart disease) [50]. Therefore, timely prescribed effective treatment of the underlying disease is the first step toward preventing the development of CHF requiring the use of diuretics, which reduces the likelihood that the doctor will face the problem of DR.

The key to the second step in the prevention of DR is understanding the multifaceted mechanisms of hypervolemia and congestion in patients with heart failure, which leads to the conclusion that diuretics should be considered only as one of the components in the complex of measures for secondary prevention of CHF, including non-drug interventions, optimal pathogen-specific combination pharmacotherapy, electrophysiological methods of treatment, surgical interventions, and the use of circulatory assist devices [1, 15, 51].

The use of diuretics should be preceded by non-drug interventions, which should be initiated already at the stage of latent CHF and continued after the appearance of signs of decompensation; the more pronounced the congestion, the more active the measures should be. Careful adherence to nutritional recommendations is perhaps the most effective and the least

costly non-pharmacological measure [1, 52]. Since observational studies suggest an association between sodium intake with fluid retention and the risk of hospitalization in patients with CHF [53–55], and sodium intake in the general population is usually high (> 4 g / day), limiting daily intake of Na^+ to the level recommended by WHO experts, equal to 2.5–3 grams (6–7 grams of table salt), is a reasonable goal for patients with moderate CHF [15, 46]. This approach prevents Na^+ retention in the postdiuretic period and is considered as a way to overcome the inhibition phenomenon [45]. For patients with DR, even more severe restriction of Na^+ intake to the level of 2 g per day is required (in this case, the help of a nutritionist is usually needed) [45]. Compliance with a diet with a more significant restriction of Na^+ in the outpatient setting is challenging, and its implementation may even significantly increase the risk of overall mortality and rehospitalizations due to exacerbation of CHF [56–58].

Limiting fluid intake to 1.5–2 liters per day is relevant only in severe CHF requiring intravenous administration of diuretics [15, 57]. It is worth noting that severe CHF is a term traditionally used in the Russian Federation to denote heart failure corresponding to stage II B [57] and functional class IV [59, 60]. With severe hypervolemic hyponatremia (Na^+ concentration in blood plasma below 125 mmol / l), more severe restriction (up to 800–1,000 ml / day) of fluid intake may be required [51]. In dilutional hyponatremia, the use of tolvaptan, i.e. a selective, competitive vasopressin V2 receptor antagonist, is indicated (the efficacy and safety of other vaptans, in particular, the non-selective V1a / V2 receptor antagonist pecavaptan [61]), without which in such a situation, effective and rapid DR management is practically impossible [62, 63].

To prevent the development of the inhibition phenomenon, diuretic therapy should be started with the lowest effective dose of the drug (preference should be given to a loop diuretic) [45]. For the same purpose, diuretics should be prescribed in combination with neurohumoral modulators (angiotensin-converting enzyme inhibitors or a combination of valsartan and sacubitril, beta-blockers, mineralocorticoid / aldosterone receptor antagonists) and sodium-glucose cotransporter-2 inhibitors (dapagliflozin or empagliflozin). Such combination therapy is also optimal in the development of late DR associated with adaptive changes in the distal nephron segments during long-term diuretic therapy [45, 51].

In the active phase of therapy, the dose of the diuretic should be gradually selected so that the excess of diuresis over the fluid taken is 1–2 l / day, with a daily body weight decrease by 0.75–1 kg. More rapid dehydration cannot be justified and only leads to hyperactivation of neurohormones and rebound fluid retention in the body [15, 45]. The strategy of using a diuretic in an intermittent mode with "shocking" diuresis once every few days is definitely flawed (every other day, once a week, etc.), as it inevitably leads to pseudo-resistance. Nevertheless, intermittent intravenous bolus administration of diuretics may be useful to maintain the euvolemic state in hemodynamically stable outpatients receiving continuous oral saluretic therapy [64]. This strategy leads to a decrease in the number of hospitalizations due to cardiac decompensation by preventing the development of resistance to oral forms of loop diuretics. In particular, in moderately decompensated patients, euvolemia may require as little as one or two doses of intravenous diuretics [64–67].

In the maintenance phase of therapy, which proceeds after reaching the euvolemic state, the dose of the diuretic can be reduced. But in any case, the latter should be above the natriuretic threshold (the steep part of the dose – response curve) providing balanced diuresis and maintaining lean body mass [45]. Like in the active phase, taking into account the pharmacokinetic features that reduce the likelihood of developing DR (high and predictable bioavailability, smooth and prolonged action with minimal postdiuretic sodium retention even with a single use during the day) and pleiotropic anti-aldosterone activity, torasemide is the drug of choice [68–70].

Patients should be taught to self-adjust diuretic doses based on monitoring of symptoms / signs of congestion and daily weight measurements [51]. In the event of a significant increase in dyspnea and edema, or an unexpected weight gain of more than 2 kg in 3 days, the patient should immediately inform their physician [51]. In such a situation, the first step in escalating therapy is to double the dose of a loop diuretic (if for some reason it has not been used before, then a dose should be equivalent to 40–80 mg of furosemide) until the effect or the maximum safe dose is reached [14, 45, 51]. At the same time, intravenous administration of a loop diuretic is preferable to its oral administration (Figure) [64].

It should be borne in mind that it is often not enough to simply increase the dose of an intravenously administered diuretic to overcome DR. With preserved kidney function, the maximum daily dose of furosemide, above which there is only a slight further increase in

natriuresis, is 80–160 mg; in patients with stage 3–4 chronic kidney disease or nephrotic syndrome, this dose is 160–240 mg [46]. Very high doses (500 mg of furosemide or more) may be required in patients with end-stage renal disease [45]. Most experts agree that shortening the intervals between diuretic administration (or their continuous infusion) allows to overcome postdiuretic sodium retention and is more effective than a single high-dose administration of the drug [9, 14]. Thus, a daily dose of a diuretic divided into two injections gives a greater effect than the same dose administered once a day, provided that both doses exceed the diuretic threshold [45].

Combination therapy with diuretics of different groups is the next step in overcoming DR after increasing the dose [14]. The effect is achieved both due to sequential blockade of the nephron and mutual potentiation of diuretic activity [21, 29]. For example, acetazolamide increases sensitivity to loop diuretics by correcting metabolic alkalosis [71, 72] and to thiazide diuretics by reducing the expression of pendrin [40].

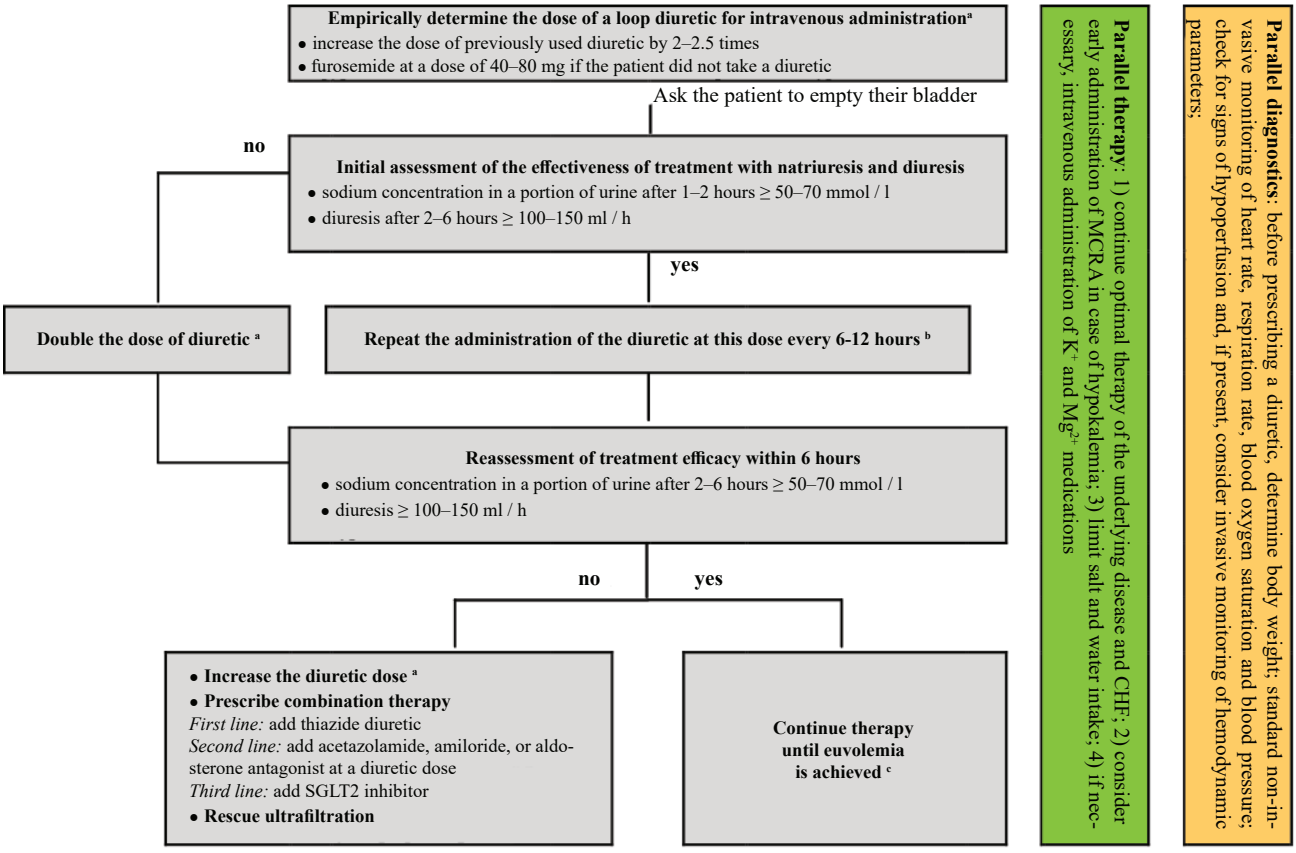

Figure. Block diagram of the use of diuretics in decompensated heart failure and resistance to diuretics [9, 14, 51].

[a] the maximum daily dose of an intravenous diuretic is a dose equivalent to 400–600 mg of furosemide (for example, bumetanide – 10–15 mg), however, in patients with severe renal dysfunction, an increase in the dose to 1,000 mg may be required; [b] in patients with good diuresis after a single administration of a diuretic, dosing once a day may be considered; [c] consider reducing the diuretic dose if daily urine output exceeds 5 liters; [d]> 50 mg / day; IV – intravenously (in the form of continuous infusion, or bolus); CHF – chronic heart failure; MCRA – mineralocorticoid receptor antagonists; BP – blood pressure; SGLT2, sodium-glucose cotransporter type 2

In addition to evaluating the effectiveness of combination therapy, careful clinical and laboratory monitoring of its safety is required. At the start of aggressive combination therapy with diuretics, which is combined with the use of modern neurohumoral modulators, some decrease in blood pressure, an increase in the level of urea and creatinine in the blood (a decrease in the estimated glomerular filtration rate), and a change in the concentration of potassium in the blood plasma are expected. Asymptomatic hypoten-

sion usually does not require any change in therapy. Faintness / slight dizziness is common and often stops over time – patients should be soothed by careful monitoring of blood pressure. An increase in creatinine by 20–30% above the baseline level is acceptable [51]. The key point of therapy in DR is that, although aggressive anti-edematous treatment is associated with deterioration in renal function, survival paradoxically improves [46, 73, 74].

The algorithm shown in the figure is fully applicable only to hemodynamically stable patients with hypervolemia demonstrating DR [14]. Clinicians are well aware that one of the main reasons preventing the use of high doses of diuretics (as well as titration of the dose of disease-modifying drugs to the target level) in patients with decompensated heart failure is systemic hypotension [75, 76]. Between 5 and 25% of patients with symptomatic CHF have low systolic blood pressure with or without signs and / or symptoms of hypoperfusion [75–78]. Symptomatic or severe asymptomatic hypotension (SBP < 90 mm Hg) may be aggravated by diuretic-induced vasodilation and hypovolemia [51].

Despite the fact that routine use of non-glycoside inotropic agents and vasoconstrictors as a tool to solve the problem of DR is not recommended [14], in a clinical situation with low cardiac output and hemodynamic instability, they cannot be avoided [51]. In this case, their use can have dramatic effectiveness and ensure DR management [36]. The arsenal of well-studied drugs and those currently under study includes vasoconstrictors (e.g., norepinephrine, midodrine, and vasopressin), inotropes with vasoconstrictive properties (e.g., dopamine, epinephrine, and droxidopa), cardiotonic agents (e.g., dobutamine, milrinone, and omecamtiv mecarbil), and inodilators, among which, according to some experts, the use of levosimendan is the most promising (the use is acceptable in the absence of a pronounced decrease in systolic blood pressure > 85 mm Hg) [51, 79–84].

If DR is associated with persistent clinically pronounced hypotension, which is figuratively called the Achilles heel of a patient with heart failure [85], short-term use of glucocorticoids may also be required, which not only contribute to an increase in blood pressure, but also have a positive effect on the functional state of the kidneys, demonstrating an increased renal response to diuretics in the experiment and in the clinical setting [85–88].

In patients with normal or elevated systemic blood pressure, a combination of diuretics with vasodilators, in particular, serelaxin (a recombinant analogue of human relaxin-2), low doses of nesiritide (a recombinant human brain natriuretic peptide), the vasopressin antagonist tolvaptan (especially in dilutional hyponatremia when an aquaretic has a significant advantage over a saluretic), and adenosine type 1 receptor antagonists (for example, aminophylline) may be effective [7, 14, 45, 89–96].

The use of drugs from the group of sodium – glucose cotransporter 2 inhibitors (for example, dapagliflozin or empagliflozin), which not only improve the prognosis in CHF, but also have diuretic and nephroprotective effects, can contribute to achieving euvolemia in refractory edema [14, 97–100].

In the presence of hypoalbuminemia (less than 35 g / l) in a patient with CHF (for example, in combination with nephrotic syndrome or liver cirrhosis), the effectiveness of diuretics is significantly reduced [15, 36]. In this case, to enhance the diuretic effect (especially at albumin levels below 25 g / l), intravenous administration of albumin immediately before diuretic therapy should be discussed [40, 101, 102], although the feasibility of this approach is not always supported by the results of clinical studies [45, 103]. Albumin should also be administered to compensate for its loss after laparocentesis (20-50 g with each procedure) with the evacuation of a large volume of ascites fluid (sometimes 4–6 liters per day are removed), which is performed in patients with CHF complicated by stable DR at the terminal stage of the disease [45].

Renal replacement therapy is the last resort for some patients with DR [9, 104]. So, one should not wonder why it is called life-saving [105]. Taking into account the fact that at the stage of deciding on renal replacement therapy, many patients have clinically pronounced hypotension (remember, the Achilles heel), it is preferable to choose sparing regimens using a minimum volume of extracorporeal blood and an ultrafiltration rate of no more than 250 ml / hour or peritoneal dialysis [79, 106–108].

It should be clearly understood that if it comes to ultrafiltration, its use does not always improve the fate of a patient with advanced heart failure. Mortality in patients with DR who need dialysis support is higher than among those patients for whom optimized pharmacotherapy was enough to achieve euvolemia (the probability of all-cause mortality is 3 times higher) [109]. Mechanical circulatory support (mono- and biventricular) can improve the prognosis in this cohort

of severe patients, often with unstable hemodynamics [79, 110–112].

Promising approaches aimed at overcoming DR, which are currently being discussed, include administration of hypertonic saline in combination with a high dose of a loop diuretic [113, 114], the subcutaneous route of furosemide infusion [115, 116], improvement of sequential nephron blockade due to the chronotherapeutic approach (for example, prescription of a thiazide drug 30 minutes before a loop diuretic) [43, 117], the use of non-neutralizing monoclonal anti-adrenomedullin antibodies, which increase its half-life and promote movement of this vasoactive peptide from the interstitium into the bloodstream (Adrecizumab) [117, 118], effects on the apelinergic system [119, 120], as well as thoracic sympathetic ganglia blockade (at the level from T6 to T11) with lidocaine [121, 122]. The results of prescribing anticoagulants in the nephrology clinic [19, 123] allow to hope that their use in DR will help restore sensitivity to loop diuretics in patients with CHF [19, 124]. Taking into account the well-known role of inflammatory mediators in the mechanisms of renal dysfunction in patients with type 1 and 2 cardiorenal syndrome, unloading of the heart and kidneys may also be useful [125-128].

CONCLUSION

The term “diuretic resistance” remains poorly defined, but it is usually considered that this is an inability to maintain natriuresis and diuresis at a level sufficient to ensure euvolemia, despite an adequate dose and regimen of loop diuretic administration. DR can develop both at the start of diuretic therapy and during their long-term use and is determined by various mechanisms. As a rule, DR reflects the progressive course of CHF and is often associated with a poor prognosis. Prevention, early detection of progression, and a set of measures aimed at overcoming DR contribute to improving the prognosis and significantly increasing the quality of patients’ life.

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Allergen-specific immunotherapy in allergic rhinitis

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ABSTRACT

The review focuses on allergen-specific immunotherapy (AIT), a treatment method for atopic diseases, including allergic rhinitis. The theoretical and practical basics, development prospects, indications and contraindications to AIT, peculiarities of AIT execution in allergic rhinitis, and tolerogenic effects of immunotherapy are considered. Advantages and disadvantages of each of the two preferable routes of allergen administration in AIT, subcutaneous and sublingual, are described. The main goals of further AIT advancement include shortening of treatment protocols with no significant loss of efficacy, creation of a safer adverse effect profile, and distribution of AIT in developing countries.

Keywords: allergen-specific immunotherapy, allergen administration routes, allergic rhinitis, allergen tolerance

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Аллерген-специфическая иммунотерапия при аллергическом рините

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РЕЗЮМЕ

Настоящий обзор фокусирован на аллерген-специфической иммунотерапии (АСИТ), методе лечения атопических болезней, включающих аллергический ринит. Рассматриваются теоретические и практические основы, перспективы развития, показания и противопоказания к АСИТ, особенности выполнения процедур АСИТ при аллергическом рините и толерогенные эффекты иммунотерапии. Отмечены преимущества и недостатки каждого из двух предпочтительных методов введения аллергенов, подкожного и подъязычного. Главной целью дальнейшего усовершенствования АСИТ является укорочение продолжительности протоколов лечения без существенной потери эффективности, создание более надежного профиля безопасности и распространение АСИТ в развивающихся странах.

Ключевые слова: аллерген-специфическая иммунотерапия, пути введения аллергенов, аллергический ринит, толерантность к аллергенам

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INTRODUCTION

Allergen-specific immunotherapy (AIT) was invented by an outstanding British researcher Leonard Noon (1877–1913) (Fig.1), who published his revolutionary article “Prophylactic inoculation against hay fever” in The Lancet in 1911 [1]. Throughout all his life, L. Noon was involved in research; he stayed at the laboratory till past midnight and often worked till three or four in the morning and sometimes till dawn. Dying from tuberculosis, he continued to think about his remarkable invention long before allergy medications appeared [2].

AIT has been used in healthcare for over 100 years, helping millions of atopic patients, children, females, and males and creating novel unexpected trends in medicine. Currently, AIT efficacy and safety have been demonstrated in multicenter, placebo-controlled, double-blind studies, and protocols of AIT execution and clinical comments to them have been approved in the international position papers [3]. AIT, the only available disease-modifying method for atopic conditions, is classified as a treatment modality with the highest level of evidence-based medicine with advantages exceeding those in pharmacotherapy. In particular, AIT can halt the allergic march in patients with allergic rhinitis [4–6].

BASICS AND FUTURE PROSPECTS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY

There are at least five described routes of allergen administration into the body: subcutaneous [7–9], sublingual [10–12], oral [13–15], epicutaneous [16, 17], and intralymphatic [18, 19]. Currently, two main, well-studied, and documented routes of AIT are being used in clinical practice: subcutaneous [7] and sublingual [10]. The third route, oral AIT, is being developed and standardized.

The main indications to AIT are atopic diseases, allergic rhinitis, allergic asthma, and atopic dermatitis. The additional indications include [20]: failure to effectively control the symptoms by pharmacotherapy; serious adverse events caused by pharmacotherapy; reluctance of patients to receive continuous or long-term pharmacotherapy.

AIT is contraindicated in relapses of atopies, uncontrolled and severe asthma, severe cardiovascular disorders, psychoses, malignant tumors, severe systemic autoimmune diseases, pregnancy at the start of immunotherapy, and acute infections [21]. Adverse events in the course of AIT are divided into systemic and local ones. Systemic adverse events occur extremely rarely and are monitored by World Allergy Organization (WAO) [22, 23].

The recommended age to start AIT ranges in different countries from 3 to 5 years [7, 20]. The efficacy and patient-relevant benefits of AIT are proven and evident [24]. Unfortunately, low compliance with treatment in some children and their parents results in violation of conventional AIT protocols and decreased treatment efficacy.

Fig. 1. Leonard Noon (1877–1913)

Subcutaneous AIT [7–9] is a classical therapeutic method for atopic diseases that has been used for a long time. However, there is an enormous potential to improve this administration route by using combinations of allergens or allergoids with biologics like omalizumab and a great number of adjuvants (recombinant allergens, hypoallergenic variants, conformational variants, deletion mutants, allergen fragments and oligomers, as well as hybrid and mosaic antigens) [25]. Although clinical developments were mainly focused on sublingual AIT, interest in the subcutaneous route of administration has increased over the past decade. However, the need for repeated injections and the risk of serious adverse events associated with subcutaneous AIT limit wide use of this method in clinical practice. In the opinion of some researchers, the future of immunotherapy may belong to sublingual AIT [26].

Sublingual AIT is currently widely used. Allergens can be administered in the form of tablets and liquid formulations (drops). Both allergen forms are administered under the tongue and held there until swallowed or spit out. The potential for development of sublingual AIT is associated with its safety, low risk of systemic adverse reactions, long-term post-treatment benefits, and a lack of necessity to visit the hospital and consult allergists frequently [10, 11]. Therefore, treatment can be quickly modified in terms of allergen composition, if necessary.

Currently, oral AIT in food allergy is extremely relevant, however, it has not been proven, whether this route really results in desensitization to food allergens. The method involves regular oral administration of small but gradually increasing amounts of food allergens. Mild adverse reactions during oral AIT are frequent, for example, mouth or throat itching and abdominal pain. Today, oral AIT has been standardized only for peanuts [13–15], but preliminary clinical trials have shown substantial benefits of this method in treating cow's milk, hen's egg, and peanut allergies.

Epicutaneous AIT is based on high density of professional antigen-presenting cells in the epidermis that are administered with an allergen for a greater impact on immunity. At the same time, it is possible to use both allergens and tolerogenic adjuvants [16]. A modification of the method consists in applying interchangeable skin patches for daily maintenance of the allergen dose [17]. Since the epidermis is not vascularized, the risk of systemic adverse events is lower than in routine AIT. Epicutaneous AIT demonstrated a more prolonged treatment effect in food allergy.

Intralymphatic AIT includes three ultrasound-guided injections of indoor, pollen, and animal allergens in the inguinal lymph nodes at 4-week intervals, making it possible to receive the entire treatment within two months. However, in total, AIT typically takes approximately three years [19]. Continuously increasing numbers of published trials on intralymphatic AIT are promising, but still insufficient for its routine use [27].

Before the start of AIT, the allergist prescribes a medication, a route of administration, and a treatment schedule. The trained nurse performs subcutaneous injections in the allergist's office [28]. After that, the patient must remain under observation for at least 30 minutes following the injection. Monthly patient visits to the hospital for these procedures are mandatory. Sublingual formulations (drops or tablets) are taken by the patient at home daily. In case of adverse events, the patient should inform the allergist about them.

The prospects of AIT include development of alternative application routes, immune-modulating adjuvants, allergoids [29, 30], recombinant vaccines [18, 31–33], and containers for allergens, such as virus-like particles and liposomes. The principal aim of AIT development is to shorten existing long protocols without a significant loss of efficacy, create a better adverse effect profile, and distribute AIT in developing countries [3].

ALLERGEN-SPECIFIC IMMUNOTHERAPY IN DIFFERENT POPULATIONS OF PATIENTS WITH ALLERGIC RHINITIS

In allergic rhinitis, AIT has been used for over 100 years, showing high efficacy. Sublingual AIT is generally recommended for treating seasonal and perennial allergic rhinitis in adults and children, with some limitations in perennial allergic rhinitis due to house dust mite (HDM) allergens [34]. However, due to heterogeneity of allergens, different approaches to publishing study reports, and a lack of the established dose, standardization of sublingual AIT in HDM sensitized patients is being approved. The study [35] reported long-term effects, which lasted for up to 7 years, after 2-year sublingual AIT in mono- and polysensitized children. In the monosensitized children, a more sustained benefit was observed.

In another study [36], the efficacy and safety of 300 index of reactivity HDM allergen extract tablets were assessed in 5–16-year-old children with allergic rhinitis in a randomized, double-blind, placebo-controlled study. The HDM sublingual tablets significantly improved symptoms of HDM-induced perennial allergic

rhinitis, caused the required immune response, and their safety profile in pediatric patients was consistent with that in adults, with no new safety concerns.

In adults with HDM-induced perennial allergic rhinitis who suffered from other atopic diseases, such as asthma, conjunctivitis, and atopic dermatitis, the sublingual AIT efficacy was studied [37]. It was demonstrated that the therapy improved not only the outcomes for allergic rhinitis, but also its comorbid conditions. A prolonged positive effect after 3-year sublingual AIT was observed in elderly patients with HDM-induced allergic rhinitis [38]. In another study [39], 41.9% of elderly patients with HDM-induced allergic rhinitis discontinued treatment within 2 years of sublingual AIT, and the most frequent reasons for that included unavailability of medications and persistent symptoms of the disease.

In the study [40], most patients with allergic rhinitis (average age – 27.3 years) were satisfied with 3-year sublingual AIT, as the therapy reduced the severity of symptoms and improved the quality of life. There has been no significant difference in the efficacy between subcutaneous and sublingual AIT in recent meta-analyses, but the sublingual route had more local adverse effects though less systemic ones [41]. The cost minimization analysis indicated that HDM tablets were a cost-minimizing alternative to subcutaneous AIT with HDM allergen extracts, when considered from a societal perspective [42]. For the treatment of persistent moderate to severe HDM-induced allergic rhinitis, HDM tablets, in addition to pharmacotherapy, had cost efficiency of Î2,276 over the 9-year time period compared with pharmacotherapy plus placebo which cost 7,519. Besides, persistent moderate to severe HDM-induced allergic rhinitis was not well controlled by allergy medications [43].

The effects of AIT on local allergic rhinitis have not been documented yet. A randomized, double-blind, placebo-controlled phase II trial was carried out that included *D. pteronyssinus* sensitized patients with local allergic rhinitis receiving subcutaneous AIT [44]. The primary markers included symptoms, medication scores, and medication-free days, whereas the secondary markers included skin testing, serum-specific IgE and IgG4, nasal allergen provocation test, and adverse events. AIT resulted in significant improvements in both primary and secondary markers versus placebo. After 12 months of the AIT, a substantial and pronounced increase in allergen tolerance with negative nasal allergen provocation test in half of the patients and significant serum-specific IgG4 were observed. The immunotherapy was well tolerated; no systemic reactions occurred. This study demonstrated that subcutaneous AIT is a safe and clinically effective treatment method for local allergic rhinitis, confirming that this disease is a new indication for AIT [44].

TOLEROGENIC EFFECTS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY

AIT is executed by administering gradually increasing doses of the causative allergen up to the maintenance dosage to achieve long-term tolerance to this allergen [45]. AIT triggers the tolerogenic immune response to the culprit allergen, resulting in the T helper 2 (Th2) cell allergen tolerance and T helper 1 (Th1) cell polarization. Therefore, allergen-specific IgE antibodies switch to IgG_4 and IgA_2 blocking antibodies [46, 47], and allergen-specific memory cells (memory Treg cells, memory T and B cells) are formed [48]. The whole spectrum of tolerogenic cells and molecules is implemented closer to mid-year [49–52], causing stimulation of Th1 cells in terms of upregulating antibody isotype switch. In individuals who respond to AIT, the IgE level is initially elevating but returning to the baseline value by the end of the first year of the immunotherapy. The IgG_4 level is rising, but an increase in IgG_4 stabilizes after the second year of the immunotherapy [51] (Fig.2).

By the end of the first year and later, the levels of immunosuppressive cytokines interleukin (IL)-10, transforming growth factor (TGF)-β, IL-27, IL-35, and IL-10-secreting Breg cells that inhibit Th2, Tfh, Th17, Th22, and ILC2 increase. It is worth noting that the level of Bregs rises earlier than that of Tregs [53], reaching the maximum after the second year of the immunotherapy [51]. However, a medium level of Tregs is observed after 30 weeks of immunotherapy; then it continues to increase, slightly declining by the end of the third year of AIT [51].

Regulatory cells (Tregs and Bregs) play an important role in formation of memory cells (memory Treg cells, memory B and T cells), which are required for long-term efficacy of AIT [48, 50, 52, 54].

CONCLUSION

AIT is disease-modifying treatment for atopic conditions, having the highest level of evidence-based medicine with advantages exceeding those in pharmacotherapy. It is essential that AIT can halt the allergic march in patients with allergic rhinitis [4–6]. AIT has more than a 100-year history after Leonard Noon invented this method. Currently, allergen quality is be-

ing improved and new medication combinations and protocols are being developed and approved, expanding research of allergen tolerance after AIT and accumulating clinical experience.

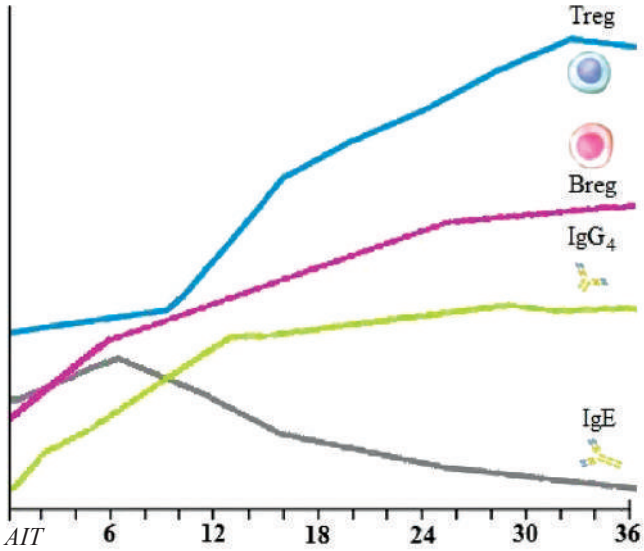

Fig. 2. Sequence of changes in immunological parameters in AIT: blocking IgG_4 antibodies increased by month 12 and stabilized after the second year, whereas IgE antibodies initially increased and diminished after the first year. Regulatory B cells (Bregs) reached a high level at the end of the second year, but regulatory T cells (pTregs) significantly increased from week 30 of the therapy, slightly declining by the end of the third year. According to [51]

AIT showed high efficacy in allergic rhinitis, making it possible to use this method in a new form of pathology, local allergic rhinitis [44]. Among routes of allergen administration, the sublingual AIT is considered preferable due to a better adverse effect profile and efficacy similar to that in subcutaneous AIT. AIT may be combined with allergy medications, including biologics, making this modality perspective in allergy [3].

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Evolution of paradigms in the study of depression: from a unitary concept to a biopsychosocial model and interdisciplinary approaches

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ABSTRACT

The review is devoted to the consideration of the history of paradigms in the study of depressive disorders in terms of modern understanding of depression in psychiatry and clinical and medical psychology and its correlation with the biopsychosocial model in medicine. The review also contains works devoted to the study of the prevalence and comorbidity of depressive disorders and their relationship with suicidal behavior. The existing limitations in the study of depressive disorders in psychiatry and clinical psychology and the issues of interdisciplinary integration and interdisciplinary barriers are considered in detail.

The review includes publications indexed in the Web of Science, Scopus, Russian Science Citation Index, and PubMed databases. Depression is a major medical and psychological problem due to its widespread prevalence in the general population, in primary care, among patients with various chronic somatic symptom disorders who receive treatment in community and specialized hospitals and clinics, and among clients of psychological centers and social services. In 1996, the Harvard T.H. Chan School of Public Health, based on the materials of the World Health Organization (WHO) and the World Bank, published estimates and prognosis for the prevalence of depressive disorders around the world. According to their data, depression in 1990 was ranked 4th in terms of the severity of the leading causes of the burden of the disease, and according to the baseline scenario of development, by 2020 it should have been ranked 2nd after coronary artery disease.

The review is focused on the need to revise the baseline scenario of development and start a new discussion on the study of depressive disorders under new conditions, such as the psychological state of society during the COVID-19 pandemic and lockdown, as well as on the eve of healthcare transition to the 11th Revision of the International Classification of Diseases.

Keywords: depression, depressive disorders, biopsychosocial model, comorbidity of depression, depression concepts, depression diagnosis, depression classification

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Эволюция парадигм в изучении депрессии: от унитарной концепции к биопсихосоциальной модели и междисциплинарным подходам

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РЕЗЮМЕ

Настоящий обзор литературы посвящен рассмотрению истории парадигм в изучении депрессивных расстройств в аспекте современного понимания депрессии в психиатрии, клинической и медицинской психологии, ее соотнесения с биопсихосоциальной моделью в медицине. В обзоре также содержатся работы, предметом которых было изучение распространенности и коморбидности депрессивных расстройств, связь с суицидальным поведением. Отдельно рассмотрены существующие ограничения в изучении депрессивных расстройств, имеющиеся в психиатрии и клинической психологии, вопросы междисциплинарной интеграции и междисциплинарных барьеров.

В обзор включены публикации, индексируемые в Web of Science, Scopus и Russian Science Citation Index, а также в базе PubMed. Депрессия является важнейшей медицинской и психологической проблемой в связи с ее широким распространением в общей популяции, в первичной медицинской сети, среди пациентов, страдающих различными хроническими соматическими заболеваниями, которые получают лечение в больницах и клиниках общего и специализированного профиля, клиентов психологических центров и социальных служб. В 1996 г. Гарвардская школа здравоохранения, основываясь на материалах Всемирной организации здравоохранения и Всемирного банка, опубликовала расчеты и прогноз распространенности депрессивных расстройств в мире. Согласно приведенным данным, депрессия в 1990 г. по тяжести ведущих причин бремени болезни занимала 4-е место, а по базовому сценарию развития к 2020 г. должна была выйти на 2-е место после ишемической болезни сердца.

Обзор ориентирован на необходимость ревизии базового сценария развития и открытия новой дискуссии по проблемам изучения депрессивных расстройств в новых условиях – психологическое состояние общества в период пандемии COVID-19 и карантинных мер, а также накануне перехода здравоохранения на 11-й пересмотр международной классификации болезней.

Ключевые слова: депрессия, депрессивные расстройства, биопсихосоциальная модель, коморбидность депрессии, концепции депрессии, диагностика депрессии, классификация депрессии

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

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INTRODUCTION

Depression was systematized and described for the first time in the nosology by E. Kraepelin [1]. He noted that manic-depressive insanity, on the one hand, covers the entire area of the so-called periodic and circular psychoses and, on the other hand, includes simple mania, most of the clinical presentations referred to as melancholia, as well as a significant number of amentia cases. It should also definitely include a light, but long-lasting, painful mood, which should be considered either as a previous stage of severe disturbances, or as a transition without clear boundaries into the area of personal predisposition. He also noted that over time he became more convinced that these clinically diverse presentations are manifestations of a single process. In addition, E. Kraepelin suggested that later, new studies would result in a number of clinical subforms of circular psychosis or separate groups of nosologies, one way or another associated with depression [1]. He believed that, if this happens,

the signs that had hitherto been brought to the forefront could become a definite measure.

The entire subsequent history of the study of depression has shown that these statements very accurately anticipated the identification of various subtypes of depression on the basis of the clinical presentations that form its symptom complexes.

CHANGES IN THE CLASSIFICATIONS OF MENTAL AND BEHAVIORAL DISORDERS

This unitary concept dominated for more than fifty years and began to be revised only in the second half of the XX century. The most significant innovations were made by K. Leonhard [2] and, somewhat later, by J. Angst and C. Perris [3] based on the study of hereditary and constitutional factors in patients with depression. Their aim was to correlate heredity and premorbid personality traits with the clinical presentation, course, and outcome of depression. These works and further changes in the taxonomy of mood disorders classified manic-depressive psychosis into a proper bipolar variant and recurrent depression. Despite this, in a later revision of the International Classification of Diseases ICD-9 [4], they were still considered as different types of the course of manic-depressive psychosis, that is, they were still assigned to the same nosology.

Large-scale changes in the classifications of mental and behavioral disorders at the end of the XX century most profoundly affected the cluster of mood disorders. Avoiding the nosological principle of building a taxonomy while maintaining the categorical approach became a compromise between the progress in clinical psychiatry and the difficulties in finding the etiology and pathogenesis of affective disorders. Thus, the era of manic-depressive psychosis was replaced by the time of the "depressive episode" [5] or "major depression" [6–10]. After fundamental changes in psychiatric classifications, their authors began to develop adapted versions for other specialties, including nursing. It contributed to closer integration with psychosomatic medicine and marked the beginning of a new milestone in psychiatry, which can be described as "general medical".

In parallel with this process, psychopharmacology of antidepressants saw a breakthrough. It was primarily associated with the emergence of drugs with a selective effect on certain neurotransmitters. The breakthrough not only tangibly changed the quality of depression therapy, but also increased the treatment effectiveness of concomitant chronic somatic symptom disorders [11]. Being a mental disorder, depression manifests itself through a number of symptoms, including a somatic component. It leads to the formation of symptoms similar to those that occur in various lesions of organs and systems, which makes diagnosis quite difficult. Meanwhile, the development of information systems to support clinical decision-making based on machine learning and knowledge in the field of diagnosis and treatment of depressive disorders has by now reached the level that can algorithmize the entire diagnostic and treatment process [12, 13]. This makes it accessible not only to psychiatrists, but to all medical and non-medical specialties and specializations involved in the diagnosis and treatment of depression. The exceptions here are cases of severe and resistant conditions, which require psychiatric qualifications and proper experience. As a rule, such patients need inpatient treatment in specialized facilities.

At the same time, information technologies remain dependent on existing clinical concepts, classification approaches, therapy standards, and clinical protocols. In this regard, the remark of I.V. Davydovsky [14] made in the middle of the XX century is still relevant. He insisted that a medical thought has a risk of drowning in particulars, getting lost in details, and, therefore, it is time to look for new concepts and new generalizing theories and ideas based on the accumulated facts. In relation to depressive disorders as very common ones, this means that the continuous search for their biological markers as an alternative for clinical diagnosis can lead to the fact that non-specific and unstable biological parameters can be introduced into diagnostic criteria. It will make refinement of the clinical classification difficult. However, limiting such a search is categorically unacceptable, since it is the introduction of laboratory and instrumental parameters into diagnostic criteria. It is one of the best opportunities to overcome the psychosomatic dualism that still exists and the psychiatric stigma that follows it, existing both in the society and medical community. These two factors continue to prevent patients from seeking psychiatric care, which results in their not receiving proper treatment. On the contrary, timely diagnosis and treatment of depression is the key to success in helping people suffering from this disorder.

Mental health services in different countries sometimes have fundamentally different organization. This concerns the issues of deinstitutionalization of mental healthcare, the number of days patients spend in hospitals, the degree of integration with psychosomatic medicine, the presence or absence of sectoral services,

and the presence or absence of general practitioners or family doctors. However, if we do not take into account population-based epidemiological studies, which are extremely few, we have some understanding of mental disorders, including depression, only from the data of requests for care. It means that the population scale of the mental health problem is either not known or is known for major mental disorders. Data on a group and risk factors are generally contradictory [15]. This fact resulted in four negative trends.

1. Lack of complete information on the number of new cases and the number of people in need of therapy.

2. Uncertainty in the outcome of cases in which patients disappeared from the sight of mental health services during treatment.

3. Impossibility to assess psychosocial impacts and adaptation of patients.

4. Self-treatment.

Similar generalizations were made somewhat earlier by G. Thornicroft et al. [16]. They focused on possible prospects for changing the current situation. For instance, the need for further transition of psychiatry in the field of general medicine was emphasized. Among other things, it would increase the availability of care to people suffering from depressive disorders, especially since they have a high level of comorbidity with somatic symptom disorders. W. Rutz et al. [17] compared the prevalence of depression, as well as the severity of symptoms in people suffering from it, with an epidemic requiring immediate intervention.

In addition to these factors, the evolution of paradigms in the study of depression was influenced by the increase in its prevalence [18], the emergence of antidepressants, their improvement with subsequent psychopharmacological research [19], reforms in mental health services [20], the integration of psychiatry with psychosomatic medicine [21], and breakthroughs in biological psychiatry [22], clinical psychology [23], and suicidology [24].

Thus, we participate in the events in which depressive disorders are studied from many different angles. It turned out that its clinical presentation in patients of mental hospitals is mostly manifested as vital depression [25], which, in turn, is a depressive syndrome in the classical sense [1]. For many decades of the last century, it was considered a manifestation of endogenous depression, but after fundamental changes in psychiatric taxonomies, primarily in the United States [6–10], the depressive syndrome was verified as major depression and considered independently. In patients who do not seek psychiatric care, the clinical presentation of depressive disorders is more variable.

Therefore, gradual departure from the unitary paradigm in understanding the clinical features of depression has made room for other concepts. Back in the 1970–80s, a number of researchers [26–29] began to differentiate depressive spectrum disorders, which, in addition to depression and its clinical variants, included mixed anxiety – depressive disorder, brief and prolonged depressive reactions, etc. Symptoms and clinical polymorphism of depression are reflected in the following concepts: the "simple depression – complex depression" ratio [30], depressive affect modality [31], a binary (two-level) typological model of depression [32] or its clinical dynamics: the concept of depressive affect evolution by stages [33]. In addition to clinical concepts, psychological concepts appeared throughout the XX century, some of which have experimental confirmation. The most well-known concepts among them are psychodynamic [34], behavioral [35], and cognitive [36], which have made an important contribution both to the understanding of the psychological mechanisms of depression development and to its non-drug therapy. Drug treatment of depressive disorders also resulted in the emergence of new paradigms. Here it is necessary to single out monoamine [37], cytokine [38], and neurotrophin [39] hypotheses, which gave impetus to the study of depression pathogenesis. The concept of responding to psychopharmacotherapy of depression [40] has become the basis for improving the quality of evaluating treatment effectiveness. The study of depression epidemiology also provides opportunities for expanding its concepts. In this regard, the theory of lower rates of depression in large urban areas of the United States [41] is of interest, which makes early studies of its prevalence in different populations relevant.

Taking the above into consideration, it becomes clear that the epidemiological rates of depression obtained in studies may differ depending on the concept that the researchers adhered to and the methodology used. Therefore, these parameters noticeably differ. At the same time, the accumulation of data on the prevalence of depressive disorders in differentiated populations provides opportunities for the emergence of more complete paradigms.

In the USA, 50% of people with major depression are managed by general practitioners and only 20% – by psychiatrists [42]. In the United Kingdom, only 10% of patients end up in a psychiatrist's office [43]. In Greece, this figure is lower than 5% [44].

These data indicate that depression is a general medical problem, not only a psychiatric one. The abovementioned does not diminish the role of psychiatry in the study of this phenomenon [45], treatment of severe [46] and resistant [47] cases, and educational activities [48]. This is one of the world's health priorities, as primary care physicians are interested in making depression screening and diagnosis as short as possible.

According to B.C. Montano [49], about one third of 20–30 patients that a doctor may see on a weekday will have depressive symptoms, and 2–3 of them will have clinical major depression. At the same time, the practice of a family doctor includes measuring blood pressure, but does not include screening for depression, even though it is as common as hypertension. The screening method involves filling out a questionnaire by the patient, which can be done while waiting for a doctor's appointment. Therefore, all patients should be screened, even if it is obvious that there is a somatic symptom disorder in the foreground.

Suffering caused by depressive disorders and somatic symptom disorders is quite pronounced and often causes psychological pain. This is a long-term unpleasant and unstable feeling, characterized by a perception of oneself as incapable and inferior, as well as by unmet psychological needs and social isolation. Psychological pain is an important aspect of depressive disorder and is associated with a higher risk of suicidal thoughts and suicidal behavior. Depression increases sensitivity to psychological and physical pain. Conversely, higher tolerance for physical pain is associated with suicidal behavior [50]. Therefore, the earliest possible diagnosis of depression is crucial to prevent suicidal behavior through timely initiation of depression therapy. It is common knowledge that depression and suicidal behavior, along with a genetic link, have a number of common psychosocial factors: low education level, low-paid work or unemployment, unstable socio-psychological situation, frustration of basic needs, losses, etc. [51]. It should also be underlined that more than 90% of people who committed suicide, had suffered from at least one mental disorder related to major psychiatry, with major depressive disorder being the most common – 56–87% [52]. It is one of the leading causes of chronic disability [53] and affects 350 million people worldwide [54].

Before moving on to the question of depression prevalence in general medicine, we will focus on the prevalence of depressive disorders in the general population, showing the baseline scenario of the problem.

PREVALENCE OF DEPRESSIVE DISORDERS IN THE WORLD

The most famous international large-scale multicenter study, which was carried out in different countries on all continents, showed the average prevalence of depression within 1 month. It amounted to 5.8% [55]. At the same time, another major prospective epidemiological study (Epidemiologic Catchment Area) of the US National Institute of Mental Health was published. According to its data, 9.5% of the US population over 18 years suffered a mood disorder within one year [56]. Another epidemiological study (National Comorbidity Survey) found that within one year, the incidence of affective disorders in US residents was equal to 11.3% [57]. On the European continent, the level of major depression was approximately 7% [58]. In 1996, the Harvard T.H. Chan School of Public Health published estimates and forecasts for the prevalence of depressive disorders around the world, based on materials from WHO and the World Bank. According to their data, in 1990, depression was ranked 4th in terms of severity of the leading causes of the disease burden, and according to the baseline development scenario, it should have been ranked 2nd after coronary artery disease by 2020 [59]. Currently, there is a need to revise the baseline scenario of development and start a new discussion on the problems of studying depressive disorders under new conditions, such as the psychological state of society during the COVID-19 pandemic and lockdown, as well as on the eve of healthcare transition to the 11th Revision of the International Classification of Diseases.

Epidemiological studies in psychiatry are considered some of its scientific foundations [60] and are traditionally extremely relevant due to high prevalence of mental and behavioral disorders, the emergence of new assessment tools, and changes in approaches, for example, a gradual transition from the categorical diagnostic approach to the dimensional one. The data obtained in epidemiological studies outline the priority tasks of psychiatry, which determines the leading research areas aimed at improving diagnosis, quality of care, and its organization. Epidemiology in psychiatry is contributing to the progressive growth of the evidence base needed to determine the cost-effectiveness of such initiatives and measures [61]. The large epidemiological studies mentioned above and many others not included in this review have marked a steady increase in the prevalence of depressive disorders. Moreover, they established the importance of

interdisciplinary research on mood disorders and created a platform for the development and integration of methods of care for patients with depression based on biological, psychological, and social approaches. It is also epidemiological studies that emphasize that there are fewer patients with depressive disorders in mental health services. This showed the need to introduce standards for their diagnosis and treatment into somatic healthcare, including primary healthcare [62].

Over the past three decades, many countries have made enough changes to provide patients in the primary care setting with access to affordable diagnosis and treatment of depression, including screening for depression and evaluation of the effectiveness and safety of ongoing therapy. Besides, due to breakthroughs in psychopharmacology, psychotherapy, and engineering technologies, the range of effective psychotropic and non-pharmacological treatment options has expanded significantly. There have been dozens of trials showing the benefits as well as cost-effectiveness of treating depression in the primary care setting and some attempts to destigmatize the mental disorder. Depression has become a medical priority along with such somatic symptom disorders as hypertension and diabetes [63].

Among patients in somatic healthcare, the prevalence of depressive disorders ranges from 9 to 66%. On the one hand, this variation is due to different frequency of depression associated with certain diseases and, on the other hand, due to different research methodologies [64]. It is illustrated by the data obtained in comorbidity studies on depression and somatic symptom disorders in different years (Table).

Table

Prevalence of depression in somatic inpatients

Diseases	Depression prevalence, %	Study
Post-stroke period	47–50	Carota, J. Bogousslavsky [65]
Traumatic brain injury	≈20	G.P. Prigatano [66]
Epilepsy	23	A.C. Viguera et al. [67]
Huntington's disease	38	A.M. Codori et al. [68]
Multiple sclerosis	18–27	R.M. Sobel et al. [69]
Postinfarction period	≈9–≈66	L. Feng et al. [70]
Coronary artery disease requiring coronary artery bypass grafting	40–50	E. Hayes et al. [71]
Coronary artery disease at an early stage	17	W. Jiang, J.R. Davidson [72]
Diabetes	9–27	N. Hermanns et al. [73]
Addison's disease	50	M. Fornaro et al. [74]
Hyperthyroidism	30	A. Suwalska et al. [75]

Table

Diseases	Depression prevalence, %	Study
Chronic pain associated with cancers	>15	M.E. Geisser et al. [76]
Pancreatic cancer	50	C.P. Carney et al. [77]
Cancer of the mouth and throat	22–40	S. Reisine et al. [78]
Colon cancer	13–25	M. Stommel et al. [79]

Based on the meta-analysis data [70], with the greatest dispersion, it can be stated that the cumulative prevalence of depression in the postinfarction period varies significantly by region, instruments used to define depression, study quality, sex, race, extent of myocardial damage, and diabetic status. Thus, this study showed that depression prevalence in any pathology can be influenced by a third disease or a number of concomitant diseases. The same study shows overall prevalence of depression in patients with myocardial infarction, which is 28.7%. In total, it is estimated that 50% of patients with cardiovascular diseases are diagnosed with mood disorders [80]. Moreover, there is evidence that depression can contribute to the development and progression of heart disease [81].

Data on depression prevalence among patients in somatic inpatient hospitals clearly show that it occurs quite often in somatic symptom disorders. Taking into account the above trends in the burden of the disease [59], there is a good reason for developing and taking urgent measures of specific psychoprophylaxis for both somatic symptom disorders and depressive disorders. This also applies to treatment of such comorbidity cases. The challenges that healthcare faces today affect not only purely clinical problems, but also raise questions of economic and humanitarian significance. This is primarily due to the COVID-19 pandemic, which has had a serious impact on the mental health of the world's population, exacerbated the course of somatic symptom disorders, and led to implementation of lockdown measures that contributed to the deterioration of mobility and accumulation of stressful experiences including those associated with a loss of beloved people, narrowed the circle of contacts, and deteriorated the availability of medical care [82].

The comorbidity of depression and somatic symptom disorders is not a homogeneous phenomenon. There are dependent and independent cases. But modern classifications [5, 10] divide them into different clinical categories. For example, depression is not considered as a reaction to a somatic symptom disorder, and symptoms typical of depression, such as poor

mood, anergy, anhedonia, insomnia, melancholy, and anxiety, are not symptoms of somatic symptom disorders [49]. The presence of somatic symptom disorders in patients with depression can be explained by mediating mechanisms, such as unhealthy lifestyle and adverse pathophysiological disorders [83]. There are alternative explanations for somatic comorbidity in people with depression: genetic pleiotropy, iatrogenic effects, and the "somatic depression" phenomenon. In the latter, the symptoms of depression are the result of clinical or subclinical somatic symptom disorders [84].

Clinical epidemiology has made a significant contribution to the understanding of comorbidity, but has not yet resulted in a common research methodology. On the one hand, this is due to a constant shift in paradigms. On the other hand, it can be explained by the diagnostic traditions adopted in different countries. This applies not only to duplicating classifications [5, 10], but also to psychometry, which in some cases is used in the form of questionnaires, in others – in the form of rating scale scores.

Disunity in diagnostic approaches also applies to medical specialties, including clinical and medical psychology, which are not in adequate demand in public health. However, it was psychology that gave impetus to the development and progress of psychological counseling and psychotherapy, including depressive disorders [85]. Medicine, for its part, has limitations in the interaction in the "doctor – patient" system. This is due to implementation of high technologies into medicine, including artificial intelligence, which depersonalizes both medical care and patients themselves [86].

Based on the presented data, it can be stated that depression is an extremely common and severe disease, often leading to disability in patients. In addition, depressive disorders tend to be chronic and recurrent [87] and occur in all age groups [88]. At the same time, the level of depression is higher in people with various types of physical [89], psychological [90] and social [91] problems. In addition, high prevalence of depressive disorders and understanding of limitations of mental health service coverage contributed to transfer of psychiatric approaches into physical medicine [92]. Therapy of depression outside traditional psychiatric services is also possible due to the emergence of new generations of antidepressants. They cause less adverse events in the course of therapy, which allows for treatment of mild depression in the outpatient setting [40].

THERAPY FOR DEPRESSION

It is also necessary to mention the opportunities for non-drug treatment of depression in the primary care. Here we are talking primarily about psychotherapy [93] and social counseling [94]. In this regard, improving the organization of care models used in general medicine for depression treatment is becoming a topical issue [95]. Summarizing the above, there is a steady trend – medical and clinical psychologists are interested in studying the conditions resulting in depressive disorders, their psychodiagnostics, as well as development and improvement of standards for psychological counseling and therapy. In turn, social workers provide necessary assistance to restore lost skills and work capacity and help in adaptation. Thus, depression is a kind of model of movement into an interdisciplinary space.

This movement is most fully reflected in the biopsychosocial model, which turned out to be relevant for depression as well [96]. The system of care within this model is much broader than the conventional approaches adopted in institutional psychiatry, where drug treatment dominates. Therefore, approaches to the organization of care for depression cannot be the only field of activity in biomedical psychiatry. This process of interdisciplinary integration has a positive effect, which is to increase availability of mental healthcare for the population. The development of a high-quality interdisciplinary approach and integrative medicine can play a serious role in creating a common understanding of a person in their somatopsychic integrity at different levels of the hierarchical organization with multiple-valued correlates.

Therefore, studying depression, conditions for its initiation, clinical course, and comorbidity is of paramount importance for modern development of public healthcare. The transition from a unitary concept of depression to a biopsychosocial model and interdisciplinary approaches both provides great opportunities for understanding it and expands the arsenal of methods for helping patients.

CONCLUSION

This review examines the background and evolution of views on the understanding of depression, its biological nature, psychological determinants, social causes, and changes in classification approaches and basic concepts. Despite the clinicians' clear understanding of significant psychological experiences and problems in their patients that meet the diagnostic criteria for clinical depression, it is not yet well recog-

nized in somatic medicine. For this reason, patients with depressive disorders often endure long-term suffering that places a heavy burden on them, their loved ones, relatives, social environment, health systems, and society. Currently, there are significant achievements in the recognition, pharmaco- and psychotherapy of depressive disorders, and the study of their etiology, pathogenesis, clinical presentation, course, and prognosis. Therefore, the most important task for specialists is to transfer the knowledge and experience accumulated on this issue by researchers in the field of mental healthcare to somatic healthcare and psychosocial rehabilitation centers in order to increase the effectiveness of these services for clients suffering from depressive disorders.

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Mechanisms of vascular aging

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ABSTRACT

Vascular aging plays a key role in morbidity and mortality in the elderly. With age, the vasculature undergoes changes characterized by endothelial dysfunction, wall thickening, decreased elongation, and arterial stiffness. The review focuses on the main cellular and molecular mechanisms of aging, including oxidative stress, endothelial dysfunction, inflammation, increased arterial stiffness, and molecular genetic aspects. Their role in the pathogenesis of diseases associated with aging is considered. Some of the molecular mechanisms underlying these processes include increased expression and activation of matrix metalloproteinases, activation of transforming growth factor β1 signaling, increased levels of C-reactive protein, interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)α, and N-terminal pro B-type natriuretic peptide (NT-pro-BNP), and activation of proinflammatory signaling pathways. These events can be caused by vasoactive agents, such as angiotensin II and endothelin-1, the levels of which increase with aging. For prevention of cardiovascular diseases, it is important to understand the mechanisms underlying age-related pathophysiological changes in the blood vessels.

Keywords: vascular aging, cardiovascular disease, oxidative stress, endothelial dysfunction

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Механизмы сосудистого старения

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РЕЗЮМЕ

Старение сосудистой системы играет ключевую роль в заболеваемости и смертности среди пожилых людей. С возрастом сосудистая сеть претерпевает изменения, характеризующиеся дисфункцией эндотелия, утолщением стенок, снижением растяжимости и артериальной жесткостью. В данном обзоре уделяется

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внимание основным клеточным и молекулярным механизмам старения, включая окислительный стресс, эндотелиальную дисфункцию, воспаление, повышенную артериальную жесткость; молекулярно-генетическим аспектам. Рассматривается их роль в патогенезе заболеваний, связанных со старением. Некоторые молекулярные механизмы, лежащие в основе этих процессов, включают повышенную экспрессию и активацию матричных металлопротеиназ, активацию передачи сигналов трансформирующего фактора роста β1, повышение концентрации С-реактивного протеина, интерлейкина-1, интерлейкина-6, фактора некроза опухоли α и натрийуретического пептида N-концевого про-В-типа, активацию провоспалительных сигнальных путей. Эти события могут быть вызваны вазоактивными агентами, такими как ангиотензин II, эндотелин-1, концентрация которых увеличивается при старении. Для профилактики сердечно-сосудистых заболеваний важно понимание механизмов, лежащих в основе возрастных патофизиологических изменений сосудов.

Ключевые слова: сосудистое старение, сердечно-сосудистые заболевания, окислительный стресс, эндотелиальная дисфункция

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

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INTRODUCTION

Cardiovascular diseases are the most common cause of death worldwide. At the same time, vascular aging is the main and irreversible risk factor for disease development [1–4]. Obviously, aging leads to certain changes that make cardiovascular system predisposed to diseases even in the absence of traditional risk factors, such as hypertension, diabetes mellitus, smoking, etc. [5]. Although aging is inevitable, gaining knowledge about the mechanisms underlying this process in the cardiovascular system has changed the perception of vascular aging as a modifiable risk factor [6]. At the molecular and cellular levels, aging is associated with structural, mechanical, and functional changes in blood vessels, characterized by increased arterial stiffness, decreased nitric oxide production, increased production of reactive oxygen species (oxidative stress), and endothelial dysfunction.

Cardiovascular aging reduces the contractile and mechanical efficiency of blood vessels. Specific changes include increased smooth muscle tone, increased collagenolytic and elastolytic activity, and arterial wall thickening. These changes contribute to an increase in systolic blood pressure, an increase in cardiac load, and systemic vascular resistance [7]. Progressive hypertrophy of cardiomyocytes, inflammation, and gradual development of cardiac fibrosis are signs of cardiac aging [8]. Age-related arterial wall remodeling contributes to the pathogenesis of vascular diseases leading to an increase in morbidity and mortality.

Thus, arterial aging is the main factor contributing to an increase in the incidence and prevalence of cardiovascular diseases, mainly due to chronic inflammation of arteries. Inflammatory signaling driven by the angiotensin II cascade causes unfavorable age-related structural and functional remodeling of arteries [9].

In order to prevent an increase in mortality from cardiovascular diseases in aging population, it is necessary to understand the mechanisms underlying age-related pathophysiological changes in blood vessels, including oxidative stress, mitochondrial dysfunction, and chronic inflammation.

ENDOTHELIAL DYSFUNCTION IN AGING

Vascular endothelium is a dynamic structure that performs many vital functions. The vascular endothelium must constantly maintain a balance between oxidants and antioxidants, vasodilators and vasoconstrictors, pro- and anti-inflammatory molecules, and

pro- and antithrombotic signals. Endothelial function in young people is regulated by traditional risk factors for cardiovascular diseases, but old age is independently associated with the development of vascular endothelial dysfunction [10]. Increased prevalence of cardiovascular diseases in aging is caused by aging of vascular endothelial cells and the associated vascular dysfunction. Aging of endothelial cells is a pathophysiological process involving functional and structural changes, such as dysregulation of vascular tone, increased endothelial permeability, arterial stiffness, impaired angiogenesis and vascular repair, and decreased mitochondrial biogenesis in endothelial cells [11].

Among various mechanisms leading to vascular dysfunction, endothelial dysfunction is one of the earliest and most important events. Impaired endothelial vasodilation is an early sign of vascular aging, preceding clinical manifestations of vascular dysfunction. It is also the first step toward cardiovascular diseases. [6]. Many pathophysiological changes in the endothelium contribute to vascular dysfunction associated with aging, such as decreased nitric oxide (NO) production and activation of calcium signaling (Ca^{2+}), increased endothelial permeability, impaired angiogenesis and vascular repair, and decreased mitochondrial biogenesis in endothelial cells. Thus, cell cycle regulation, oxidative stress, altered Ca^{2+} signaling, and vascular inflammation are involved in the pathophysiological process [11]. In addition, the accumulation of genetic damage changes normal expression and activity of genes, which leads to cellular senescence and vascular dysfunction [11]. Both macrovascular and microvascular endothelial dysfunction are the key markers of endothelial health and independent predictors of cardiovascular risk in the elderly [12].

OXIDATIVE STRESS IN AGING

Oxidative stress is involved in the pathology of many human diseases. Oxidative stress is recognized as a major factor in the pathophysiology and pathogenesis of such age-related diseases as metabolic syndrome, atherosclerosis, osteoporosis, obesity, dementia, diabetes, cancer, and arthritis [7, 13, 14]. Constant formation of free radicals, mainly reactive oxygen species (ROS), is the main characteristic of all living systems that use oxygen for their metabolism. The most common ROS are superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2), which can stimulate sequential reactions causing further free radical production and associated oxidative damage to cellular components. Oxidative stress and inflammation induce endothelial dysfunction resulting from decreased NO bioavailability [10]. Oxidative stress is involved in the pathogenesis of arterial stiffness, since oxidative damage can lead to increased vascular inflammation and cell proliferation, which can subsequently result in impaired arterial elasticity [15]. Oxidative stress in endothelial cells increases with age. It is caused by increased production of intracellular enzymes (NADPH oxidase and endothelial NO synthase (eNOS)), as well as by mitochondrial respiration in the absence of a corresponding increase in antioxidant defense regulated by appropriate transcription factors [10].

VASCULAR INFLAMMATION AND MARKERS OF INFLAMMATION IN AGING

Aging is associated with chronic low-grade inflammation (sterile inflammation), i.e. a type of inflammation caused by mechanical trauma, ischemia, and stress. Chronic inflammation is associated with many pathological conditions related to aging, for example, atherosclerosis, Alzheimer's disease, etc. [16]. Inflammation is characterized by increased expression of inflammatory cytokines, adhesion molecules, and endothelial cell chemokines. Aging is associated with increased levels of circulating cytokines and proinflammatory markers. Age-related changes in the immune system, known as immune aging, and increased secretion of cytokines by adipose tissue are the main causes of chronic inflammation [17]. Chronic inflammation is thought to be related to dysfunction of immune cells, such as cell migration and signaling of pattern recognition receptors (PRRs), which are necessary to respond to pathogens. This immune dysregulation can affect conditions associated with chronic inflammation (atherosclerosis and Alzheimer's disease). The mechanisms underlying this inflammation seem to include changes in the number and function of innate immune cells and PRR activation by endogenous ligands which leads to cytokine secretion [18].

In addition, a proinflammatory response is associated with activation of nuclear factor-kappa B (NF-κB) signaling, which is an important nuclear transcription factor that promotes expression of inflammatory cytokines in endothelial dysfunction and cardiovascular diseases [10]. Alarmins mediating sterile inflammation contribute to aging. At the same time, activation of metalloproteinase-2 (MMP-2) is worth noting. It is responsible for S100A9 alarmin degradation, which limits signals that cause inflammation [16].

An age-related increase in such inflammatory peptide biomarkers as interleukin (IL)-6, IL-1, tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) [19] is one the most studied markers of aging. Higher plasma concentrations of such inflammatory factors as IL-6 and TNF-α were associated with lower muscle mass and lower muscle strength (smaller muscle area, lower appendicular muscle mass with lower grip strength). It demonstrated a relationship between immune and functional status in the body of an elderly person [20]. CRP was associated with all causes and mortality, and IL-6 was found to be a predictor of mortality [21, 22]. However, when studying markers of inflammation, it turned out that centenarians have fewer signs of inflammation [23, 24]. Inflammatory peptides are either absent or lower in centenarians compared with younger cohorts, while the levels of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β, are increased [25].

Adipokines, such as adiponectin, leptin, and visfatin, are regulators of inflammation [26]. It is interesting that adiponectin concentration changes with age and is associated with age-related health effects [27]. In the study including healthy elderly people aged 69–79 years, higher adiponectin levels were associated with an increased risk of overall and cardiovascular mortality [28].

Traditionally, N-terminal pro B-type natriuretic peptide (NT-pro-BNP) and troponin have been associated with myocardial injury and heart failure. NT-pro-BNP measurements provide predictive information about mortality and serious cardiovascular events in addition to traditional risk factors. NT-pro-BNP was a stronger biomarker of cardiovascular diseases and death risk than CRP in non-hospitalized individuals aged 50–89 years [29]. The study of 4,979 respondents (2,567 men and 2,412 women) who were divided into six age groups concluded that age over 70 years and male sex were associated with increased levels of NT-pro-BNP (> 400 pg / ml) (odds ratio (OR) 1.41; 95% confidence interval (CI) 1.20–1.65 for males) [30]. Despite their reliability as predictors of cardiac damage and cardiovascular diseases, both NT-pro-BNP and troponin increase with age, which successfully characterizes them as biomarkers of human aging [31].

INCREASED ARTERIAL STIFFNESS IN AGING

Increased arterial stiffness is an independent predictor of cardiovascular diseases that does not depend on blood pressure. Blood vessels undergo structural and functional changes characterized by remodeling (thickening) of the arteries, vascular fibrosis, and stiffness, which are manifested in aging and hypertension. Arterial stiffness is common and occurs in over 60% of people over 70 years of age and is a major independent predictor of serious cardiovascular events [32].

Arterial stiffness is assessed by pulse wave velocity measurement, pulse wave analysis, arterial stiffness analysis using 24-hour ambulatory blood pressure monitoring, and endothelial function assessment. Aortic stiffness causes an increase in pulse wave velocity and early reflection of waves with increased central hemodynamic load, which leads to damage to small arteries [33]. Patients with early vascular aging are at an increased risk of developing cardiovascular diseases. Its main component is arterial stiffness measured by an increased carotid-femoral pulse wave velocity [34].

Profibrotic processes play a significant role in the development of vascular stiffness. Fibrosis occurs in both large and small arteries. In large vessels, arterial stiffness leads to hemodynamic damage to peripheral tissues, which results in impaired endothelial function and increased vasomotor tone [32].

At the molecular and cellular levels, vascular aging and vascular changes are associated with increased expression and activation of matrix metalloproteinases (MMP), activation of transforming growth factor-b1 signaling, activation of galectin-3, and activation of proinflammatory and profibrotic signaling pathways. These events can be caused by such vasoactive agents as angiotensin II, endothelin-1 (ET-1), and aldosterone, the number of which increases with age [32].

THE ROLE OF MATRIX METALLOPROTEINASES IN VASCULAR REMODELING

In healthy vessels, deposition and exchange of extracellular matrix proteins are regulated, and the collagen / elastin ratio remains relatively constant. The imbalance of these processes leads to excessive deposition of extracellular matrix proteins, especially collagen and fibronectin, which contributes to vascular fibrosis and arterial stiffness in aging [32]. Extracellular matrix proteins are regulated with the help of metalloproteinases. In turn, they are activated by many factors associated with aging, such as interleukins, growth factors, and vasoactive agents.

Activated MMPs are capable of destroying collagen, elastin, and other extracellular matrix proteins, leading to aging and atherosclerotic effects in the arterial wall, for example, fibrosis, calcification, en-

dothelial dysfunction, and increased intima – media thickness, which further affects vascular remodeling and arterial stiffness [35–37]. Arterial remodeling mediated by MMP activation is a histopathological feature of aging arteries, hypertension, and atherosclerosis [35]. An imbalance between the activity of MMPs and their endogenous tissue inhibitors (TIMPs), which are produced by various cell types, including fibroblasts and macrophages, is important for extracellular matrix remodeling and arterial stiffness [15]. MMP-1 contributes to aging of endothelial cells through p53 activation [38].

In ischemic cardiomyopathy with progressing heart failure, there are mainly processes of collagen destruction in the extracellular matrix. They are accompanied by an increased MMP-1 level [39]. MMP-2 plays a major role in degradation of the extracellular matrix, supporting both angiogenesis and apoptosis of endothelial cells. An intermediate MMP-2 form supports survival and migration of the cells, while a fully active MMP-2 form leads to endothelial cell death. The p38 apoptotic pathway enhances synthesis and activation of the intermediate MMP-2 form. Caspases enhance synthesis and complete activation of MMP-2, but decrease the intermediate MMP-2 form [40]. In addition, MMP-2 promotes platelet aggregation and thrombus formation in response to arterial damage, and inactivation of the MMP-2 gene prevents thrombosis caused by weak stimuli in mice [41]. When studying age-related changes in MMP activity in animal models, a decrease in MMP-2 activity and an increase in MMP-9 activity with increasing age were found [42].

When studying the pathophysiology of aging in humans using the example of a healthy population of different ages, including centenarians (≥ 95 years), it was found that the MMP-2 serum activity is increased in centenarians compared with younger subjects. The authors suggested that the observed increase in MMP-2 in old age may play a positive role in achieving longevity [43].

MMP-9 is a major mediator of increased stiffness in the aging left ventricle. Aging is associated with increased MMP-9 expression in the left ventricle and decreased cardiac function. [44]. Elevated blood levels of MMP-9 and MCP-1 are positively correlated with an increase in end-diastolic volume, indicating that MCP-1 and MMP-9 are potential circulating biomarkers of cardiac aging. Increased density of macrophages in the left ventricle and stable co-localization of MMP-9 in macrophages indicate that macrophages are the main source of MMP-9 in the left ventricle and that they might provide the main inflammatory mechanism of cardiac aging [45].

MOLECULAR GENETIC PREDICTORS OF AGING

Aging can be described as a multifactorial process involving complex interactions between biological and molecular mechanisms [2]. The ability to distinguish between normal biological aging and impaired health is important. There is little experimental evidence in this area. Providing accurate indicators or predictors of ill health, as well as the ability to characterize age-appropriate optimal health, remains an important goal [2].

Aging is a major risk factor for almost all noncommunicable diseases, including cardiovascular diseases, cancer, diabetes, etc. Suggested mechanisms that contribute to aging and the development of these chronic age-related diseases include DNA damage, mitochondrial dysfunction, changes in gene expression and non-coding RNA, genotoxicity, oxidative stress, and telomere shortening [46–48]. It is known that the production of ROS by mitochondria accumulates throughout life, which leads to a state of chronic oxidative stress in old age. Since the mechanisms of antioxidant defense and the ability to repair DNA in the elderly are apparently impaired, DNA damage is considered to be a consequence of aging [49].

Impaired DNA stability is closely associated with age-related diseases. At the age of 60, chromosome lesions cease to accumulate, but in people over 85 years, the frequency of such lesions decreases [50]. Telomeres are shortened due to cell division and oxidative stress and are lengthened due to telomerase and DNA exchange in mitosis. Longer telomeres and higher telomerase activity contribute to genome stability and DNA integrity. Short telomeres are an indicator of oxidative stress and a biomarker of aging [51].

Several genetic pathways are involved in aging. A large number of microRNAs (miRs) are expressed differently during aging [52]. MiRs are found to be stable molecules even in blood serum; therefore, they are considered as promising markers in the clinical setting. Moreover, age and gender can influence the pattern of circulating miRs. [53]. MiRs are also important post-transcriptional regulators of gene expression in skeletal muscle and are associated with aging. MiRs play an important role in age-related changes in mass, composition, and function of skeletal muscles [54, 55].

Blood level of miR-126-3p significantly increases with age. It was significantly higher in the oldest

subjects compared with the youngest healthy subjects (<45 versus >75 years; relative expression: 0.27 ± 0.29 versus 0.48 ± 0.39, $p = 0.047$) [56]. Moreover, some miRs can serve as circulating prognostic biomarkers of cardiovascular aging [57].

PROTEOMIC STUDIES IN VASCULAR AGING

Structural and functional changes occur in aging vessels. They are reflected in the proteome of constituent cell types. Advances in proteomics technologies have made it possible to analyze the amount of proteins associated with the natural history of aortic aging. These changes reflect the molecular and cellular mechanisms of aging and may provide an opportunity to predict vascular health [58]. The characteristics of age-related arterial remodeling include thickening of the aortic wall, increased vascular stiffness, endothelial dysfunction, increased proliferation (invasion and / or secretion of vascular smooth muscle cells), fragmentation of elastic fibers, and collagen deposition.

Proinflammatory arterial remodeling develops with age in both humans and animals. Remodeling leads to changes in the levels of key regulatory proteins involved in pathophysiological processes. The angiotensin II signaling pathway is central in this process. Numerous protein molecules in the angiotensin II signaling pathway are activated and influence vascular remodeling in aging and associated diseases [58].

To identify biomarkers associated with aging, blood samples from 1,890 people aged 18–82 years were analyzed (1,136 men and 754 women) using MALDI-TOF mass spectrometry. The study identified 44 peptides the concentration of which differed in different age groups. The concentration of apolipoprotein A-I (ApoA1) gradually increased between 18 and 50 years of age, the levels of fibrinogen α decreased during the same age period, while albumin significantly degraded in middle-aged people. In addition, the levels of fibrinogen, albumin, and ApoA1 are closely correlated with age [1].

Comparison of young and old rats resulted in the identification of 18 peptides, whose levels vary significantly with age. Analysis of transcription and translation showed that the levels of mRNA and MFG-E8 protein (Milk Fat Globule Protein-Epidermal Growth Factor-8) in the aorta increase with age. Dual immunolabeling shows that MFG-E8 colocalizes with both angiotensin II and monocytic chemoattractant protein-1 in vascular smooth muscle cells of the thickened and aging aortic wall [59].

CONCLUSION

It is known that cardiovascular diseases associated with atherosclerosis are the main cause of morbidity, disability, and mortality in developed countries. Steady aging of the population is considered to be one of the causes of morbidity. The accumulated data indicate that arterial stiffness, arterial wall remodeling, and endothelial dysfunction are independent risk factors for cardiovascular diseases in the elderly. Traditional cardiovascular risk factors, such as high blood pressure, dyslipidemia, obesity, diabetes mellitus, smoking, etc., interact with age-related changes and contribute to the activation of atherosclerotic process. Therefore, in order to prevent the development and growth of cardiovascular diseases in the elderly population, it is necessary to understand the mechanisms underlying age-related pathophysiological changes in blood vessels.

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The role of surfactant proteins SP-A and SP-D in viral infection: a focus on COVID-19

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ABSTRACT

An immune response to invasion of viral pathogens is an integral part of maintaining the physiological functioning of the bronchopulmonary system and effective gas exchange. Collagen-containing C-type lectins (lung collectins) are some of the key proteins in the identification of viral particles. They have image-recognizing receptors that identify pathogen-associated molecular patterns, particularly viral glycoproteins. The surfactant proteins SP-A and SP-D, which are composed of trimerized units, belong to pulmonary collectins and oligomerize into higher-order structures. These proteins play an essential role in recognition and elimination of microbial pathogens (viruses, bacteria, fungi, parasites, nanoparticles, allergens) through a variety of mechanisms.

Taking into account the burden of the novel coronavirus infection caused by the SARS-CoV-2 virus, it is important to consider the role of the surfactant proteins SP-A and SP-D in the pathogenesis of the immune response to viral invasion. Currently, there are data on the direct relationship between surfactant proteins and viruses belonging to the Coronaviridae family. The SP-A and SP-D proteins modulate inflammatory responses and cytokine synthesis, but prevent an excessive inflammatory response (cytokine storm). There is also an assumption that SARS-CoV-2 directly suppresses and alters the production of surfactant proteins. Thus, the key pathogenetic role of the surfactant proteins SP-A and SP-D in the response to the viral pathogen SARS-CoV-2 is evident. Today, this is a promising area of translational medicine, which will contribute to a profound understanding of the pathogenesis of coronavirus infection for assessing the diagnostic and prognostic potentials of the surfactant proteins SP-A and SP-D in COVID-19. Additionally, it will help evaluate the therapeutic potential of recombinant fragments of human SP-A and SP-D.

Keywords: surfactant, surfactant protein A, surfactant protein D, biomarker, viral infection, coronavirus infection, COVID-19, SARS-CoV-2

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Роль белков сурфактанта SP-A и SP-D при вирусной инфекции, фокус на COVID-19

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РЕЗЮМЕ

Неотъемлемой частью поддержания физиологического функционирования бронхолегочной системы и эффективного газообмена является иммунологический ответ на инвазию вирусных патогенов. Одними из ключевых белков, участвующих в идентификации вирусных частиц, являются представители семейства коллагенсодержащих лектинов типа С (легочные коллектины). Они обладают образ-распознающими рецепторами, которые идентифицируют ассоциированные с патогенами молекулярные паттерны, в частности, вирусные гликопротеины. К легочным коллектинам относятся белки сурфактанта SP-A и SP-D, которые состоят из тримеризованных единиц и олигомеризуются в структуры более высокого порядка. Эти белки играют ключевую роль в распознавании и элиминации микробных патогенов (вирусов, бактерий, грибов, паразитов, наночастиц, аллергенов) посредством разнообразных механизмов.

С учетом бремени пандемии новой коронавирусной инфекции, вызванной SARS-CoV-2, крайне важно обратить внимание на роль белков сурфактанта SP-A и SP-D в патогенезе ответа на данную вирусную инвазию. В настоящее время известны указания на непосредственное взаимодействие белков сурфактанта и вирусов, принадлежащих к семейству Coronaviridae. Белки SP-A и SP-D модулируют воспалительные реакции и синтез цитокинов, при этом предотвращая чрезмерную воспалительную реакцию (цитокиновый шторм). Также существует предположение, что непосредственно SARS-CoV-2 подавляет и изменяет выработку белков сурфактанта. Таким образом, очевидна патогенетическая ключевая роль белков сурфактанта SP-A и SP-D в ответе на вирусный патоген SARS-CoV-2. Это на сегодняшний день является перспективным направлением трансляционной медицины как с точки зрения детального понимания патогенеза коронавирусной инфекции для оценки диагностических и прогностических потенциалов белков сурфактанта SP-A и SP-D при COVID-19, так и с точки зрения терапевтического потенциала рекомбинантных фрагментов человеческих SP-A и SP-D.

Ключевые слова: сурфактант, сурфактантный белок А, сурфактантный белок D, биомаркер, вирусная инфекция, коронавирусная инфекция, COVID19, SARS-CoV-2

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

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INTRODUCTION

An immune response to invasion of viral pathogens is an integral part of maintaining the physiological functioning of the bronchopulmonary system and effective gas exchange. Collagen-containing C-type lectins, also known as lung collectins, are among the key proteins involved in identification of viral particles. They have image-recognizing receptors that identify pathogen-associated molecular patterns, in particular,

viral glycoproteins [1]. Lung collectins include surfactant proteins SP-A and SP-D, which consist of trimerized units and oligomerize into higher-order structures [2]. These proteins play a key role in recognition and elimination of microbial pathogens (viruses, bacteria, fungi, parasites, nanoparticles, allergens) through a variety of mechanisms [2, 3]. Given the burden of the novel coronavirus infection pandemic caused by SARS-CoV-2, it is extremely important to draw attention to the role of SP-A and SP-D in the pathogenesis of the immune response to this viral invasion. Firstly, in viral diffuse alveolar damage with microangiopathy, SP-A and SP-D modulate inflammatory responses and cytokine synthesis (acting as a proactive link between innate and adaptive immunities), while preventing an excessive inflammatory response (cytokine storm) [4]. Secondly, there are indications of direct interactions between surfactant proteins and viruses belonging to the Coronaviridae family according to the classical pattern [5]. Thirdly, there is an assumption that it is SARS-CoV-2 that not only suppresses the production of surfactant proteins [6], but also causes the production of an altered surfactant [7]. This is determined by binding of the crystal-like structure of the receptor-binding domain (RBD) of SARS-CoV-2 spike protein to angiotensin-converting enzyme 2 (ACE-2) of alveolar epithelial type II cells [8], which directly produce SP-A and SP-D [1].

Therefore, despite few studies on the role of surfactant proteins SP-A and SP-D in the novel coronavirus infection, their crucial pathogenetic role in the immune response to the viral pathogen SARS-CoV-2 is obvious. Currently, it is a promising area in terms of a detailed understanding of the pathogenesis of coronavirus infection and the resulting prognostic potentials of surfactant proteins SP-A and SP-D in COVID-19, as well as in terms of the therapeutic potential of recombinant molecules SP-A and SP-D.

MOLECULAR STRUCTURE AND FUNCTIONS OF SP-A AND SP-D SURFACTANT PROTEINS

Lung surfactant is a lipoprotein complex of the respiratory mucosa consisting of 90% lipids (mainly phospholipids) and 10% proteins: SP-A, SP-B, SP-C, and SP-D [3, 4]. As mentioned above, the surfactant is predominantly produced by cube-shaped alveolar type II cells synthesizing surface active substances from typical organelles called lamellar bodies [9]. Surfactant proteins SP-B and SP-C are small hydrophobic peptides involved in packaging and processing of the surface active substance and contributing to its biophysical properties [10].

On the contrary, surfactant proteins SP-A and SP-D are large, soluble, hydrophilic proteins that are expressed on most mucosal surfaces and play the key multifunctional role in the immune response to pathogen invasion and in pulmonary immune homeostasis [1]. As stated above, SP-A and SP-D are calcium-dependent (C-type) lectins with collagen areas belonging to the group of proteins called collectins. Collectins are oligomerized proteins consisting of trimerized units with 3 polypeptide chains [11]. Each chain has a collagen triple helix domain consisting of repeating Gly-X-Y triplets, α-helical neck, and C-terminal end containing a C-type lectin or a CRD (Fig. 1) [2].

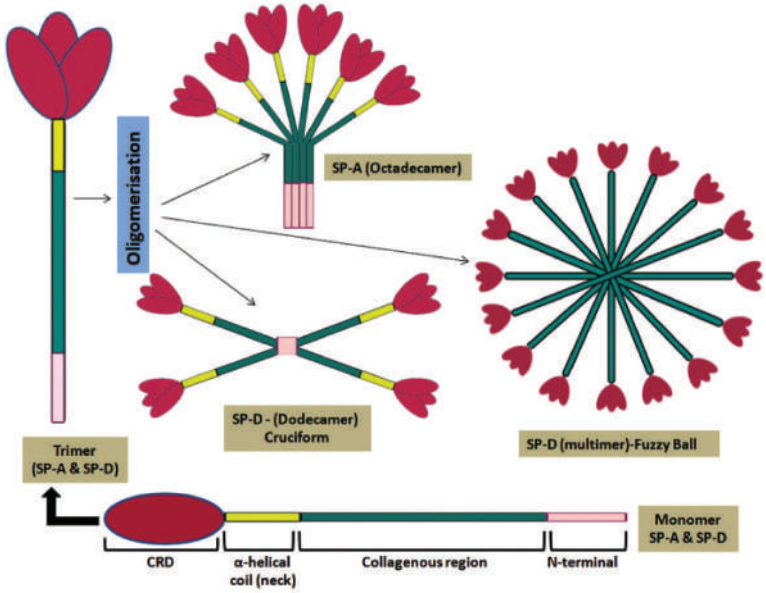

Fig. 1. Structure of SP-A and SP-D surfactant proteins (extracted from: Yasmin H., Kishore U. Biological Activities of SP-A and SP-D Against Extracellular and Intracellular Pathogens. *The Collectin Protein Family and Its Multiple Biological Activities*. 2021;103–133. DOI: 10.1007/978-3-030-67048-1_5)

Through interaction of N-terminal domains, these trimerized units oligomerize into an octodecameric structure for SP-A, forming a 630 kDa molecule consisting of 18 chains, and a dodecametric structure for SP-D forming a 520 kDa molecule, which may further amass composing "fuzzy balls" and (or) "astral bodies" [12]. This multimerization enhances the overall binding avidity to carbohydrate targets and increases the ability to agglutinate pathogens. While the SP-D trimer is a monogenic unit, SP-A is formed from two genetic products, SP-A1 and SP-A2, which have some functional differences [13].

The surfactant proteins SP-A and SP-D perform numerous functions of innate and adaptive immunity during pathogen invasion into the bronchopulmonary system (Fig. 2) [1].

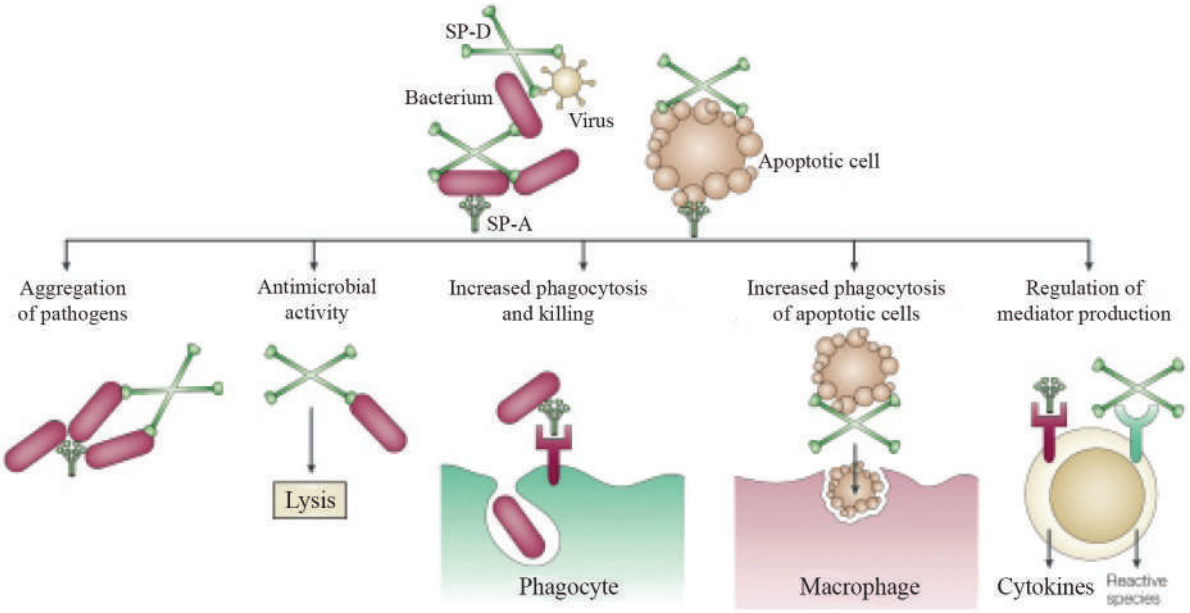

Fig. 2. Immune functions of surfactant proteins SP-A and SP-D (extracted from: Wright J.R. Immunoregulatory functions of surfactant proteins. *Nat. Rev. Immunol*. 2005;5(1):5868. DOI: 10.1038/nri1528.)

The surfactant proteins SP-A and SP-D bind and opsonize viruses, bacteria, worms, and allergens (including pollen and nanoparticles) [14]. SP-A and SP-D enhance microbial phagocytosis by such innate immune cells as macrophages and neutrophils by opsonising and aggregating bacteria and viruses, acting as a ligand for activation, and regulating the expression of surface receptors of immune cells responsible for pathogen recognition [1]. Both proteins have direct bactericidal effects against bacteria and fungi [15]. In addition, SP-A and SP-D also facilitate phagocytosis of apoptotic cells by innate immune cells and provide context-dependent regulation of cytokine and free radical production. For example, SP-A inhibits lipopolysaccharide-stimulated nitric oxide (NO) production by alveolar macrophages collected from healthy lungs, but promotes production of NO in macrophages activated by IFN-γ [14, 15].

SP-A and SP-D connect innate and adaptive immunities for regulation of protection against the background of pathogen invasion into the bronchopulmonary system. Despite the fact that both SP-A and SP-D can bind directly to T-cells and inhibit proliferation, SP-A can also inhibit proliferation of T-cells indirectly through suppressing maturation of dendritic cells (DCs) [15]. SP-D has been shown to enhance absorption and presentation of the antigen [1, 14]. *In vitro* results show that the combined role of SP-A and SP-D consists in modulation of the pulmonary immune environment in order to protect the body while preventing an excessive inflammatory response that could potentially damage the alveolar – capillary membrane and impair gas exchange, as in the case of hyper-induction of proinflammatory cytokines in development of cytokine storm as a response to SARS-CoV-2 invasion [16].

INTERACTION BETWEEN SURFACTANT PROTEINS SP-A AND SP-D AND VIRUSES

Further, the ways of specific interaction between surfactant proteins SP-A and SP-D and various viral particles will be considered, with a more detailed

description of the interaction between proteins and coronaviruses. Currently, the association between surfactant proteins and trimerized and glycosylated proteins on the surface of such viral capsids as SARS-CoV, SARS-CoV-2, respiratory syncytial virus (RSV), human immunodeficiency virus (HIV), and influenza A virus (IAV) is being actively studied. There is an assumption that surfactant proteins SP-A and SP-D have undergone coevolution with these viruses for their neutralisation through binding to glycosylated proteins for viral attachment, making their binding to the host cell impossible [14].

This interaction also enhances their aggregation, opsonisation, and clearance by phagocytes. Many enveloped viruses express class I fusion proteins, in particular, SARS-CoV-2 has spike protein (S) or S protein [17], paramyxoviruses have homotrimeric F protein, and influenza, Ebola virus, HIV, etc. – other class I fusion proteins [1, 14]. Fusion proteins of IAV and RSV are represented by a trimer with three copies of the same protein [18]. The HIV fusion envelope protein consists of two non-covalently associated glycoproteins (120 kDa and 41 kDa) – gp120 and gp41, respectively [19]. The discussed fusogenic proteins have a trimeric configuration similar to that of SP-A and SP-D, which makes it possible to suggest their coevolution for provision of selective binding to these viral surface molecules [14]. This configuration of surfactant proteins SP-A and SP-D provides direct binding with fusion proteins of the aforementioned viruses for their neutralisation, as well as for further aggregation and elimination of the virus using several fusion sites per molecule.

Annually, influenza A viruses lead to high prevalence of respiratory infection with excessive mortality in a number of cases [20]. The interaction between surfactant proteins SP-A and SP-D and influenza viruses has been investigated quite extensively. The SP-D protein binds to high-mannose oligosaccharides in immediate proximity to sialic acid binding sites to influenza hemagglutinin, which neutralizes it spatially via inhibiting its attachment to host cells [21]. On the contrary, influenza A virus demonstrates calcium-independent binding to the sialylated asparagine 187 residue of SP-A protein, making the binding of the virus to sialylated receptors impossible [22, 23]. It was also confirmed that SP-A protein is involved in the phagocytosis of influenza A virus by alveolar macrophages by using sialic acid residues as opsonin [23]. Influenza A virus may impair development of the respiratory burst of neutrophils in response to the viral infection, which leads to degranulation and intracellular destruction of bacteria by phagocytes, thus increasing the susceptibility of the human to bacterial superinfections, which is an important factor in mortality during the seasonal influenza pandemics [2]. Surfactant protein SP-D significantly potentiates the respiratory burst of neutrophils in response to influenza A virus *in vitro*, thus demonstrating a proinflammatory response [24].

Respiratory syncytial viruses (RSV) are the main causes of lower respiratory tract infection in newborns and children [25]. RSV is the leading cause of bronchiolitis and infant hospitalization in developed countries [26]. It has been shown that genetic polymorphisms in SP-A and SP-D genes are associated with susceptibility to severe RSV infection, which emphasizes their importance in the immune response to RSV [27]. It has been experimentally demonstrated that SP-A$^{-/-}$ and SP-D$^{-/-}$ mice have both a reduced capacity for RSV clearance and an increased inflammatory response in the lungs [28]. Currently, the mechanism of interaction between SP-A and SP-D surfactant proteins and RSV has not been fully studied. RSV has two main surface glycoproteins: protein G, important for attachment of the virus to the host cell, and protein F, a trimeric class I fusion protein important for fusion between the virus and the host cell membrane.

In one study, SP-A was shown to bind *in vitro* to RSV F protein, but not to G protein [29]. However, another study has shown that SP-A demonstrates calcium-independent binding to RSV using G protein, neutralizing the virus and increasing the clearance *in vivo* [30]. It has been experimentally demonstrated that a recombinant trimeric fragment of a closely-related molecule (surfactant protein SP-D) retains many functions of the native protein, while the importance of the oligomeric structure of SP-A in its interaction with RSV has not been determined [26]. Therefore, the mechanism through which SP-A and SP-D bind to RSV remains unclear and requires further investigation. However, considering the experimental efficacy of both rfhSP-A and rfhSP-D in RSV neutralization, it seems likely that virus neutralization occurs via binding of SP-A and SP-D proteins to the RSV fusion protein, which is also a trimeric protein with a helical conformation [31].

Human immunodeficiency virus (HIV) is one of the most severe public health problems around the globe [32]. HIV glycoprotein (gp)120 is required for infiltration of the virus into the cells and is the main target for HIV binding to various type-C lectins [33].

At present, the ability of surfactant proteins SP-A and SP-D to bind trimerized gp120 has been shown [34, 35]. SP-A binding inhibits the binding of CD4 using gp120, emphasizing the potential role of SP-A in HIV neutralization through blocking CD4-mediated fusion [35]. SP-D binding to gp120 prevents DC-SIGN interaction (membrane protein, C-type lectin receptor of macrophages and dendritic cells), which has also been demonstrated, to some extent, for SP-A [34, 36]. However, surfactant proteins SP-A and SP-D did not prevent binding of cyanovirin to gp120 in the experiment, which possibly confirms the DC-SIGN – gp120-type binding of SP-A and SP-D to gp120, during which none of the high-mannose N-linked glycosylation sites is responsible for DC-SIGN binding [34]. This is in contrast with cyanovirin, which is neutralized through targeting of a certain set of N-linked glycosylations [34, 35]. It is also important that SP-D binds gp41, which is required for formation of thee gp120 trimer and facilitates fusion between the viral and cell membranes [37].

Since both SP-A and SP-D have been identified in the female urogenital system, binding of these proteins to gp120 may be important in HIV infection (as a primary infection site) and in the lungs (as a common reservoir of HIV infection) [38, 39]. This binding has been shown to neutralize HIV and prevent direct infection of CD4+ of PM1 cells. However, SP-A and SP-D promote infection of immature monocyte-derived DCs and their transmission to the CD4+ T-cells in case of their co-cultivation [34, 35]. Currently, the mechanism by which collectins enhance this transmission is unclear. Further effort related to investigation of the interaction between SP-A and SP-D and HIV is of great importance in elucidating the role of these proteins in HIV infection. A number of studies have suggested that functional recombinant fragments of surfactant proteins SP-A and SP-D may have therapeutic potential for preventing infection and spread of HIV [37, 40].

THE ROLE OF SURFACTANT PROTEINS SP-A AND SP-D IN CORONAVIRUS INFECTION

Before the emergence of severe acute respiratory syndrome, SARS (SARS-CoV), in 2003, approximately ten coronaviruses in humans and animals were known. Then, the discovery of the civet cat and bat SARS-CoV viruses, as well as human coronaviruses NL63 and HKU1, followed. Currently, around 40 representatives of this family of diseases are known [41]. The sudden first pandemic of atypical pneumonia (Middle East respiratory syndrome coronavirus, MERS (MERS-CoV), 2012), its high mortality, a quick repeated outbreak after one year, and economic losses, as well as a subsequent outbreak of MERS led to extensive research on epidemiological, clinical, pathological, immunological, virological, and other fundamental scientific aspects of the group of coronaviruses. Following the outbreak of SARS and MERS, a global pandemic of another viral disease called COVID-19, induced by betacoronavirus SARS-CoV-2, is now observed [42]. Based on data available to date, COVID-19 is not much different from SARS in its clinical features. However, its mortality rate is 2.3%, which is lower than that of SARS (9.5%) and much lower than that of MERS (34.4%). However, COVID-19 can spread much more easily in the community than MERS and SARS [43].

MERS-CoV, SARS-CoV-2, and SARS-CoV are RNA viruses, the latter two containing the largest genomes among all RNA viruses [44, 45]. Genomic RNA of SARS-CoV-2 is 26.4–31.7 kb in size [44], being possibly the largest among all known RNA viruses [46]. The SARS-CoV-2 genome is similar to that of the SARS-like virus ZC45 (bat-SL-CoVZC45, MG772933.1) by over 85%. Together, these types of viruses form the unique Orthocoronavirinae subfamily with another SARS-like virus ZXC21 in the *Sarbecovirus* subgenus.

SARS-CoV-2 has a genomic structure similar to that of other betacoronaviruses. Like other coronaviruses, its genome contains 14 open reading frames (ORF) encoding 27 proteins. ORF1 and ORF2 at the 5’ end encode 15 non-structural proteins important for replication of the virus [47, 48]. The 3’ end of the genome encodes structural proteins, namely, the spike protein (S), the envelope protein (E), the membrane protein (M), and the nucleocapsid (N), as well as eight auxiliary proteins [48]. The viruses SARS-CoV and SARS-CoV-2 have differences in the spike protein (S) – the presence of the furin-like cleavage site in SARS-CoV-2 facilitates priming of the spike protein (S) and may increase the effectiveness of SARS-CoV-2 transmission in comparison with other betacoronaviruses [49]. The spike protein (S) is a class I fusion protein and is trimerized.

The conducted research showed that the majority of proteins in SARS-CoV-2 are highly homologous (95–100%) with proteins of SARS-CoV virus, which indicates their evolutionary similarity. However, two proteins (orf8 and orf10) in SARS-CoV-2 do not have homologous proteins in SARS-CoV [50]. The protein

orf8 in SARS-CoV-2 does not contain a known functional domain that activates intracellular stress signaling pathways and NOD-like receptors of NLRP3 inflammasomes [51]. This makes the analysis of the biological function of these two specific proteins (orf8 and orf10) clinically relevant.

Pathogenic coronaviruses SARS-CoV, MERS-CoV, and SARS-CoV-2 use the ACE2 receptor for access, infestation, and destruction of the alveolar lining layer and type II pneumocytes producing surfactant proteins [8]. In general, pathological features of COVID-19 resemble those observed in SARS and MERS in many aspects [52]. Biopsy and post-mortem samples in COVID-19 reveal diffuse alveolar damage, protein leakage, inflammation in alveolar walls, and desquamation of type II pneumocytes, which is typical of acute respiratory distress syndrome (ARDS) [53]. ARDS manifests through a decline in activity of proteins of the pulmonary surfactant and a change in its content [54].

In COVID-19, depletion of the pulmonary surfactant may also occur through virus-induced lysis of type II pneumocytes with associated formation of the hyaline membrane [53]. Pathophysiological data of such severely ill adult patients resemble primary surfactant deficiency in preterm infants with ARDS, successful therapy of which involves application of exogenous surfactant preparations [55]. Regarding the aforementioned, a number of studies related to the use of exogenous pulmonary surfactant in SARS-CoV-2-associated ARDS have been published [56]. These studies confirm the active role of surfactant proteins in the pathogenesis of COVID-associated severe lung damage and make it possible to consider therapeutic application of recombinant surfactant protein molecules.

However, currently, the studies devoted to specific mechanisms of interaction between SP-A and SP-D surfactant proteins and SARS-CoV-2 are few. Further, we will provide a more detailed description of the spike protein (S) participating in fusion between SARS-CoV and SARS-CoV-2 for analysis of its possible interactions with surfactant proteins (SP-A and SP-D), since it is this trimerized protein that is involved in virus neutralization and its further aggregation and elimination. It is worth noting that, despite not structurally identical fusion of SARS-CoV and SARS-CoV-2, the fusion of subunits eventually results into trimeric structures with three C-terminal ends being outside the central N-terminal trimeric core [57].

The spike protein (S) of SARS-CoV-2 has two functional subunits that mediate the infiltration of coronavirus [58]. The subunit S1 is responsible for binding to host cell receptors via the receptor-binding domain [59]. Binding to the receptor provides conformational changes in the subunit S2, which allows the fusion peptide to penetrate into the membrane of the host cell [57]. HR1 or the heptad region 1 located in the subunit S2 forms a homotrimeric orientation exposing three highly conserved hydrophobic grooves on the external surface that make it possible to bind to the heptad repeat 2 (HR2). Further, in the fusion process, a six-helix bundle (6 HB) is formed, which facilitates the approach of viral and cell membranes for viral fusion and penetration into target cells using the ACE2 receptor [8].

It is the trimeric structure of 6 HB that characterizes class I fusion proteins, which the surfactant protein SP-D is known to interact with. SP-D demonstrated this binding to recombinant trimerized proteins of SARS-CoV. The binding is calcium-dependent and inhibited by maltose, showing the properties of classical lectin – carbohydrate interaction [5]. In the meantime, a serum collectin, mannan-binding lectin (MBL), did not show the revealed binding to the purified S-protein of SARS-CoV in the experiment. It is worth noting that there are ligand differences between the collectins, and this interaction is particularly specific for SP-D [14].

Y.P. Wu et al. (2009) demonstrated that monitoring the SP-D systemic level is informative for monitoring alveolar integrity in SARS pneumonia, as well as revealed a significant correlation between plasma SP-D levels and specific antibodies to SARS-CoV, which once again confirms the role of SP-D in the relationship between innate and adaptive immunity in the pathogen invasion in the bronchopulmonary system [60]. Considering high homology of the spike protein (S) of SARS-CoV with that of SARS-CoV-2 (76.42%, according to J. Xu et al., 2020), it is possible to assume a similar classical lectin – carbohydrate binding mechanism involving calcium and multimerization of the full-length protein for facilitation of virus elimination [17].

In this context, the study by M.H. Hsieh et al. (2021) aimed at investigating a probable defensive role of the recombinant fragment of human SP-D (rfhSP-D) against SARS-CoV-2 infection is relevant. Dose-dependent binding of rfhSP-D to the spike protein S1 of SARS-CoV-2 and its RBD was demonstrated [61].

It is important to outline that the study showed that rfhSP-D inhibited the interaction between the S1

protein and the cell culture overexpressing the human ACE2 receptor. The defensive role of rfhSP-D against SARS-CoV-2 infection as an inhibitor of penetration was additionally confirmed via application of pseudotyped lentiviral particles expressing S1 protein of SARS-CoV-2 [61]. It is obvious that surfactant protein SP-D plays one of the key roles in the response to pathogen invasion of coronavirus. Considering the stated above, single studies on the potential role of SP-D as a marker for predicting the outcome and the possibility of treating COVID-19 have already emerged [62].

As for the surfactant protein SP-A, it is believed that its virus-neutralizing activity is lower than that of SP-D. However, as already mentioned, the protein plays an important role in the innate immune response to different viruses [63]. Currently, there are only a few studies devoted to the type of interaction between SP-A and the group of coronaviruses. For example, C.J. Funk et al. (2012) demonstrated that both SP-A and SP-D bind to the HCoV-229E strain and inhibit viral infection of human bronchial epithelial cells (16HBE) [64]. It can be assumed that the interaction between the surfactant protein SP-A and SARS-CoV-2 occurs through the direct interaction between the lectin domain and the molecule of glycosylated protein on the surface of the virus. Multiple direct interactions of SP-A protein with trimerized proteins, including class I fusion proteins, have been demonstrated. For example, SP-A has been noted to bind to herpes simplex virus [65], which also has trimerized surface proteins similar to those of influenza and RSV discussed earlier. The interaction between SP-A and SARS-CoV-2 has not been studied, but it probably depends both on the glycosylation status of SP-A protein itself and on the functional variants of SP-A1 and SP-A2 in humans.

A considerable amount of available data suggests that innate immune proteins SP-A1 and SP-A2 play at least an indirect role in COVID-19 infection. Thus, the functional features of SP-A1 and SP-A2 impact susceptibility to coinfections against the background of COVID-19 and the presence or absence of severe complications resulting from COVID-19 in patients with one or more non-SARS-CoV-2 pathogens [66]. Approximately 26% of COVID-19 patients have been noted to be infected with other pathogens, with RSV being one of the most frequent ones. It has been shown that it is SP-A that increases RSV clearance and that the functional trimeric fragment of SP-A is highly efficient in reducing RSV infection specifically [26]. It is also extremely important that the association between genetic variants of SP-A and ARDS development has been demonstrated [27].

SP-A variants are known to exert differentiated effects on regulation and functioning of macrophages, which is extremely important against the background of a hyperergic immune response in COVID-19 patients, indicating macrophage activation. Thus, M. Roschewski et al. (2020) put forward a hypothesis that innate immunity is involved in activation of inflammatory processes in macrophages in the pathogenesis of COVID-19 due to hyperergic inflammation, which shares common characteristics with the macrophage activation syndrome [16]. It is quite possible that the magnitude of the inflammatory response will vary depending on the SP-A genotype, which has been shown to have differentiated effects on a number of processes in the alveoli, alveolar macrophages, and epithelial cells [66].

Therefore, trimerized surfactant proteins SP-A and SP-D achieve high binding affinity to the ligand with repetitive surface structure, which is an advantage in binding to viral proteins. Additionally, these proteins have broad selectivity, allowing them to recognize a large number of rapidly changing pathogens, structurally similar trimerized fusion proteins in particular, including the spike protein (S) of coronaviruses. This prevents their attachment to the host cell, neutralizing the virus and increasing its clearance from the body, simultaneously modulating adaptive immunity. Currently, the leading role of surfactant proteins SP-A and SP-D is obvious not only in the immune response of the bronchopulmonary system, but also in the overall response of the body to the pathogen (in particular, viral) invasion.

Therefore, it is obvious that surfactant proteins SP-A and SP-D play one of the key roles in the pathogenesis of coronavirus infection. This, considering the burden of the COVID-19 pandemic, provides prerequisites for the detailed analysis of the interaction between these proteins and SARS-CoV-2, as well as for the analysis of the systemic role of SP-A and SP-D.

PROSPECTS IN APPLICATION OF SURFACTANT PROTEINS SP-A AND SP-D IN COVID-19

In conclusion, we would like to draw attention to a number of key points that justify a more in-depth and detailed analysis of the role of surfactant proteins SP-A and SP-D in the pathogenesis of COVID-19. First, surfactant proteins SP-A and

SP-D, having high binding affinity to the ligand, interact with the spike protein (S) of coronaviruses during the initial contact with the pathogen. Second, infection and destruction of type II pneumocytes producing surfactant proteins take place specifically against the background of SARS-CoV-2 invasion through the ACE2 receptor, which affects synthesis, secretion, and function of SP-A and SP-D. Third, the unique combined role of SP-A and SP-D in modulation of a cascade of the immune response consists in preventing an excessive inflammatory response that could potentially damage the alveolar – capillary membrane and impair gas exchange, as in the case of hyper-induction of proinflammatory cytokines in the novel coronavirus infection. The role of SP-A and SP-D in keeping the lungs in the hyporeactive state is essential because aberrant inflammation may rapidly affect vitally important gas exchange in the lungs through a thin alveolar – capillary membrane. Fourth, SP-A and SP-D participate in the development of severe life-threatening complications, which are accompanied by impairment of the alveolar – capillary membrane permeability.

It is important to assess the diagnostic and prognostic potential of SP-A and SP-D in coronavirus infection. Currently, surfactant proteins SP-A and SP-D are used as diagnostic and prognostic markers in many acute diseases of the bronchopulmonary system, such as community-acquired pneumonia [4, 9, 11, 14], ARDS [9, 54–56], cystic fibrosis [4, 36], interstitial lung disease [1, 4, 36], and lung cancer [1, 9]. In addition, they play an important role in modulation of chronic lung diseases and (or) bronchopulmonary dysplasia [1, 4, 9]. Over the past decade, the extrapulmonary systemic function of these proteins has been actively studied.

CONCLUSION

One of the most promising areas is evaluating the therapeutic potential of recombinant SP-A and SP-D molecules in anti-inflammatory therapy of different diseases, especially those of an infectious nature [14]. It is promising to develop forms of recombinant SP-D with alteration of the neck domain and CRD with increased binding affinity to SARS-CoV-2, as it was carried out for the molecule of mutant trimeric SP-D with increased binding affinity to influenza A virus [67]. Therefore, there is a potential for developing different forms of recombinant molecules of surfactant proteins SP-A and SP-D for treatment of COVID-19.

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Successful treatment of a severe course of coronavirus infection in the obese polymorbid patient after bariatric surgery

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ABSTRACT

We presented a clinical case of the successful treatment of a severe course of polysegmental pneumonia caused by the novel coronavirus infection, that developed in the postoperative period after bariatric surgery in the patient with morbid obesity, comorbid type 2 diabetes mellitus, ischemic heart disease, arterial hypertension, pulmonary embolism (in past medical history), and stage 3 chronic obstructive pulmonary disease.

The given clinical case demonstrates the possibility of successful treatment of coronavirus infection in the polymorbid patient at an extremely high risk of an unfavorable outcome, given timely diagnosis, combination therapy using drugs that block cytokine storm, and strict adherence to clinical recommendations.

Keywords: morbid obesity, coronavirus infection, bariatric surgery, cytokine storm, tocilizumab

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Успешное лечение тяжелого течения коронавирусной инфекции у поликоморбидного пациента с ожирением после бариатрической операции

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РЕЗЮМЕ

Представлено клиническое наблюдение успешного лечения тяжелого течения полисегментарной пневмонии коронавирусной этиологии, развившейся в послеоперационном периоде бариатрической операции у пациента с морбидным ожирением, сопутствующим сахарным диабетом 2-го типа, ишемической болезнью сердца, гипертонической болезнью, тромбоэмболией мелких ветвей легочной артерии в анамнезе, хронической обструктивной болезнью легких 3-й степени.

Приведенный клинический случай демонстрирует возможность успешного лечения коронавирусной инфекции у поликоморбидного пациента с крайне высоким риском неблагоприятного исхода при условии своевременной диагностики, комплексного лечения с использованием препаратов, блокирующих цитокиновый шторм, и строгого выполнения клинических рекомендаций.

Ключевые слова: морбидное ожирение, коронавирусная инфекция, бариатрическая хирургия, цитокиновый шторм, тоцилизумаб

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. Получено письменное согласие пациента. Исследование одобрено локальным этическим комитетом НИИКЭЛ – филиалом ИЦиГ СО РАН.

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INTRODUCTION

The COVID-19 pandemic has been a huge shock, but we should not forget about another epidemic of the 21st century – obesity. An obese patient is always a polymorbid patient. As a rule, their diagnosis includes: type 2 diabetes mellitus, arterial hypertension, hypertriglyceridemia or dyslipidemia, obstructive sleep apnea, and non-alcoholic fatty liver disease. Since the beginning of the pandemic, obesity has been regarded as a predictor of a high risk of developing severe pneumonia, acute respiratory distress syndrome (ARDS), thromboembolic complications (TEC), and death [1–4].

In obesity, the springboard for viral damage is an initially elevated level of proinflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, C-reactive protein (CRP), and ferritin, which leads to a higher risk of developing cytokine storm, ultimately leading to ARDS, shock, and rapid deterioration [4–8]. Activation of the coagulation cascade in COVID-19 in obese patients has a higher risk of fatal thromboembolic complications against the background of hypercoagulability [7]. Obesity leads to a decrease in the reserves of the respiratory system, microatelectases, violation of the ventilation / perfusion ratio, dysfunction and limited excursions of the diaphragm, and an increase in the work of the respiratory muscles with a rise in oxygen consumption. Timely identification of signs of an increased inflammatory response and inclusion of drugs that block cytokine storm in complex therapy are the key to successful treatment. These drugs include, in particular, glucocorticoids, inhibitors of IL-6 and IL-1β receptors, and Janus kinase inhibitors [8, 9].

CLINICAL CASE

Patient P., 58 years old, was admitted for bariatric surgery on 12.10.2020 to the surgical department of a clinic with a diagnosis of morbid obesity (BMI 44.4 kg / m^2), exogenous constitutional type. Obstructive sleep

apnea syndrome. Type 2 diabetes mellitus. The target HbA1c level was less than 6.5%. Diabetic sensorimotor peripheral polyneuropathy. Cardiovascular autonomic neuropathy in diabetes. Diabetic macroangiopathy (atherosclerosis of the brachiocephalic arteries). Ischemic heart disease. Postinfarction cardiosclerosis (of unknown age). Stage III hypertension, stage II arterial hypertension, risk group 4. Pulmonary embolism (small branches, 2008). Stage I chronic heart failure (CHF). Functional class I (NYHA). Dyslipidemia. Stage 3 chronic obstructive pulmonary disease (COPD), emphysematous type, severe course, group C (with frequent exacerbations), stable condition. Type 2 respiratory failure. Compensated chronic cor pulmonale. Varicose veins of the lower extremities, CEAP class 2. Gastroesophageal reflux disease (GERD), without exacerbation. Polymerase chain reaction (PCR) to detect SARS-CoV-2 in the pharyngeal and nasal swabs upon admission was negative.

On 13.10.2020, laparoscopic mini-gastric bypass was performed. The early postoperative period was uneventful; the patient was activated, the intake of fluids and nutrition was started according to the postoperative protocol for bariatric interventions, antibiotic prophylaxis with cefazolin 3 g / day and TEC prophylaxis with enoxaparin sodium 0.4 ml / day were carried out. On 17.10.2020, a rise in temperature to 38.5 °C, without symptoms of catarrh, and a decrease in oxygen saturation to 89–90% were recorded. The patient's condition was regarded as moderate. Nasal and oropharyngeal swabs were taken for SARS-CoV-2 detection using PCR.

The patient was transferred to the intensive care unit. A blood gas test revealed hypoxemia (P_{O2} 62 mm Hg) compensated by hyperventilation (pH 7.48, P_{CO2} 29.1 mm Hg, BEecf 2 mmol / l). Insufflation of humidified oxygen via a face mask at a rate of 3 l / min was started, antibacterial therapy included cefoperazone + sulbactam 2.0 g / day and levofloxacin 1.0 g / day; prevention of thrombus formation was achieved by enoxaparin sodium 0.8 ml / day; dexamethasone 24 mg / day; inhaled bronchodilator therapy was continued (berodual 2 times / day, budesonide 2 times / day, Spiolto Respimat 2 times / day); gastroprotectors – esomeprazole 40 mg / day, correction of hyperglycemia with short-acting insulin.

Taking into account pronounced abdominal obesity, the patient was lying alternately on the right and left sides, in a position close to the prone position. At the slightest improvement in the patient's condition, attempts to activate and improve the motor regime were resumed. Taking into account the increase in hypoproteinemia, the standard diet for the bariatric patient was supplemented with Nutrison Advanced Diason via sip feeding. Intravenous fluids were limited to saline to dilute the administered drugs.

On 19.10.2020, positive PCR results for SARS-CoV-2 were obtained in the oropharyngeal and nasal swabs taken on 17.10.2020. Computed tomography was not performed for technical reasons, X-ray revealed no deterioration. A competing diagnosis was made: novel coronavirus infection COVID-19, confirmed by PCR, severe course.

From 23.10.2020, there was an increase in respiratory failure and a rise in oxygen demand up to 8–10 l / min. From 25.10.2020, there was increased dyspnea, a feeling of breathlessness, a decrease in Sp_{O2} to 83% against the background of oxygen insufflation of 15 l / min. Increasing hypoxemia (Pa_{O2} 58–60 mm Hg, oxygenation index 125–130) was observed. The patient was in a clear consciousness and adequate. Hemodynamic parameters were characterized by a tendency toward hypotension. During X-ray control of 25.10.2020, bilateral polysegmental pneumonia was noted, multiple areas of veil-like ground-glass opacity were observed in all lung fields. Laboratory tests revealed signs of cytokine storm – the progression of lymphopenia, an increase in the levels of C-reactive protein and ferritin. The levels of procalcitonin, troponin I, and brain natriuretic peptide were within the reference values. The diagnosis included a complication of the competing disease: Bilateral polysegmental pneumonia. ARDS. Acute respiratory failure.

Taking into account the clinical presentation, the initial status of the patient, and signs of increasing cytokine storm on 25.10.2020, an infusion of tocilizumab 560 mg (4 mg / kg) was performed. There was a short-term improvement immediately after the infusion. On 26.10.2020, there was a resumption of dyspnea upon minimal physical exertion, Sp_{O2} 87–89% against the background of oxygen insufflation 15 l / min, and further increase in arterial hypoxemia (Pa_{O2} up to 55 mm Hg, oxygenation index 100–110). Repeated infusion of tocilizumab at the same dose was performed; antibacterial therapy was changed to imipenem + cilastatin 3 g / day and vancomycin 2 g / day. Under the control of coagulation tests and thromboelastography indices, the dose of enoxaparin was increased to 1.6 ml / day.

Against this background, a decrease in the manifestations of respiratory failure was noted: there was a significant decrease in dyspnea and oxygen demand,

from 30.10.2020, the patient did not require oxygen support, and improvement in laboratory parameters was also observed. On 30.10.2020, due to a re-positive PCR result for SARS-CoV-2 (sampling on 27.10.2020), etiotropic therapy with favipiravir 1,800 mg 2 times / day on the 1st day, then 800 mg 2 times / day was started. The changes in the main clinical and laboratory parameters are presented in Table.

Table

Dynamics of clinical and laboratory parameters

Parameter	19.10	21.10	23.10	25.10	26.10	27.10	28.10	29.10	31.10	02.11
Leukocytes, 10^9 / l (4.00–10.00)	4.17	5.21	8.93	9.30	5.86	5.37	5.92	5.81	7.46	5.32
Lymphocytes, % (20–40)	16.6	13.1	2.3	5.5	9.3	8.4	10.4	11.9	6.8	9.9
Lymphocytes, *10^9 / l (0.8–4.0)	0.7	0.68	0.2	0.52	0.55	0.45	0.61	0.69	0.51	0.53
Platelets, *10^9 / l (100–300)	181	183	216	265	279	339	372	334	348	300
ESR, mm / h	15	21	29	31	38	27	24	18	7	7
CRP, mg / l (0–5)	34.1	34.9	43.9	72.7	91.6	33.3	12.9	5.4	2.1	0.8
Ferritin, µg / l (20–250)	281.7	317.8	343.0	379.8	465.4	460.3	374.1	330	348.3	316.8
D-dimer, ng / ml (200–443)	365	273	244	283	349	394	319	302	336	267
Fibrinogen, g / l (2.0–4.0)	5.8	4.3	4.9	5.3	5.1	4.1	3.8	3.5	2.9	2.4
SpO_2, %, minimum	91	87	86	81	87	86	89	90	93	94
Respiratory rate, maximum	18	19	16	28	26	20	20	23	18	16

On 03.11.2020, the patient was transferred from the intensive care unit to the observation unit. The PCR result of 05.11.2020 was negative. The patient was discharged from the clinic on 09.11.2020.

CONCLUSION

This clinical case demonstrates the possibility of successfully treating severe coronavirus infection in a patient with an extremely high risk of an unfavorable outcome. Timely diagnosis, targeted pathogen-specific therapy, strict adherence to clinical guidelines, and timely administration of tocilizumab in combination with dexamethasone allowed to prevent fatal decompensation of respiratory failure and avoid transfer to mechanical ventilation. The prescription of antibiotic therapy, despite the negative bacterial cultures of sputum and blood, was justified given the concomitant diabetes mellitus and COPD, on the one hand, and massive immunosuppressive therapy, on the other.

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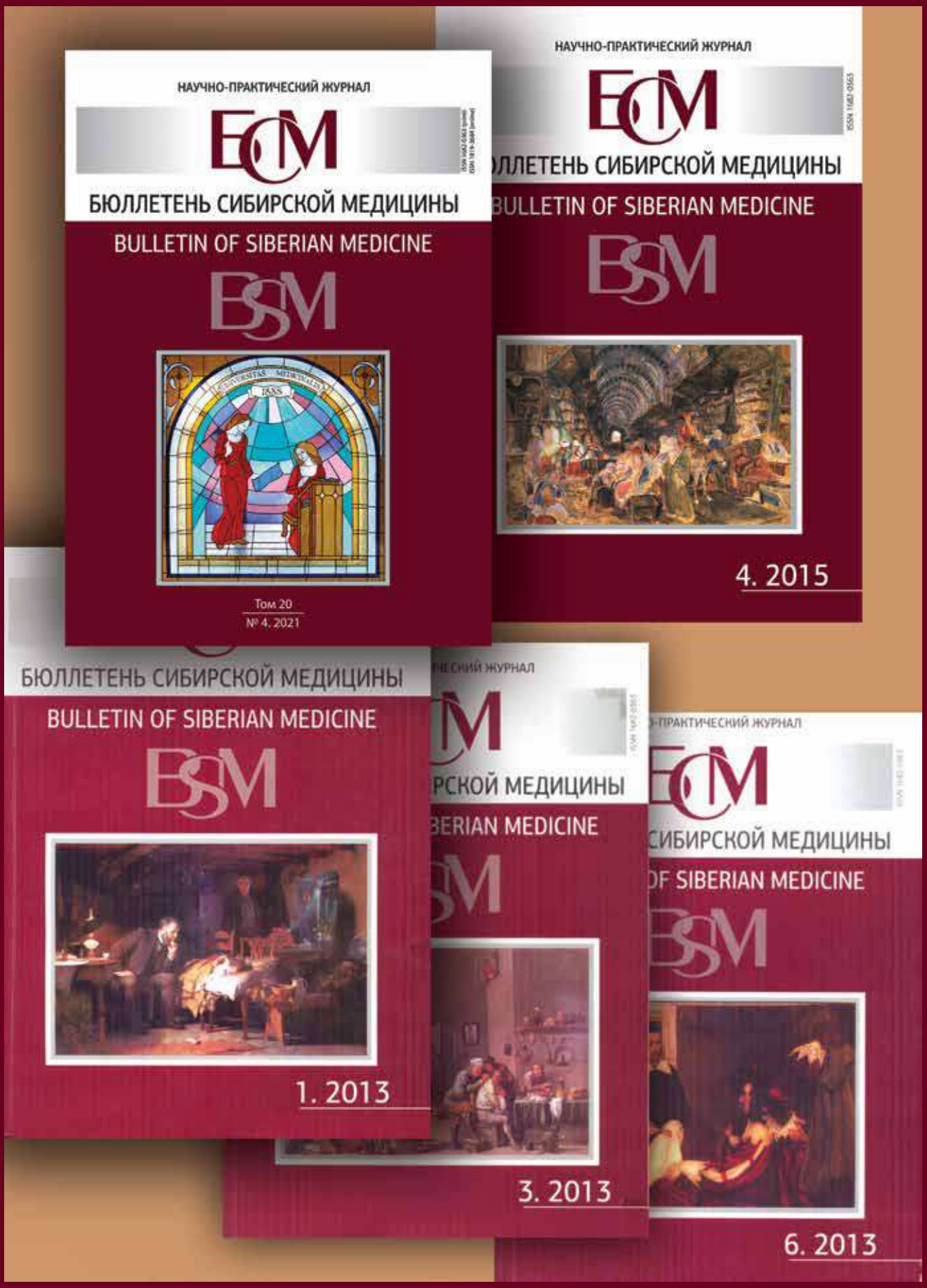

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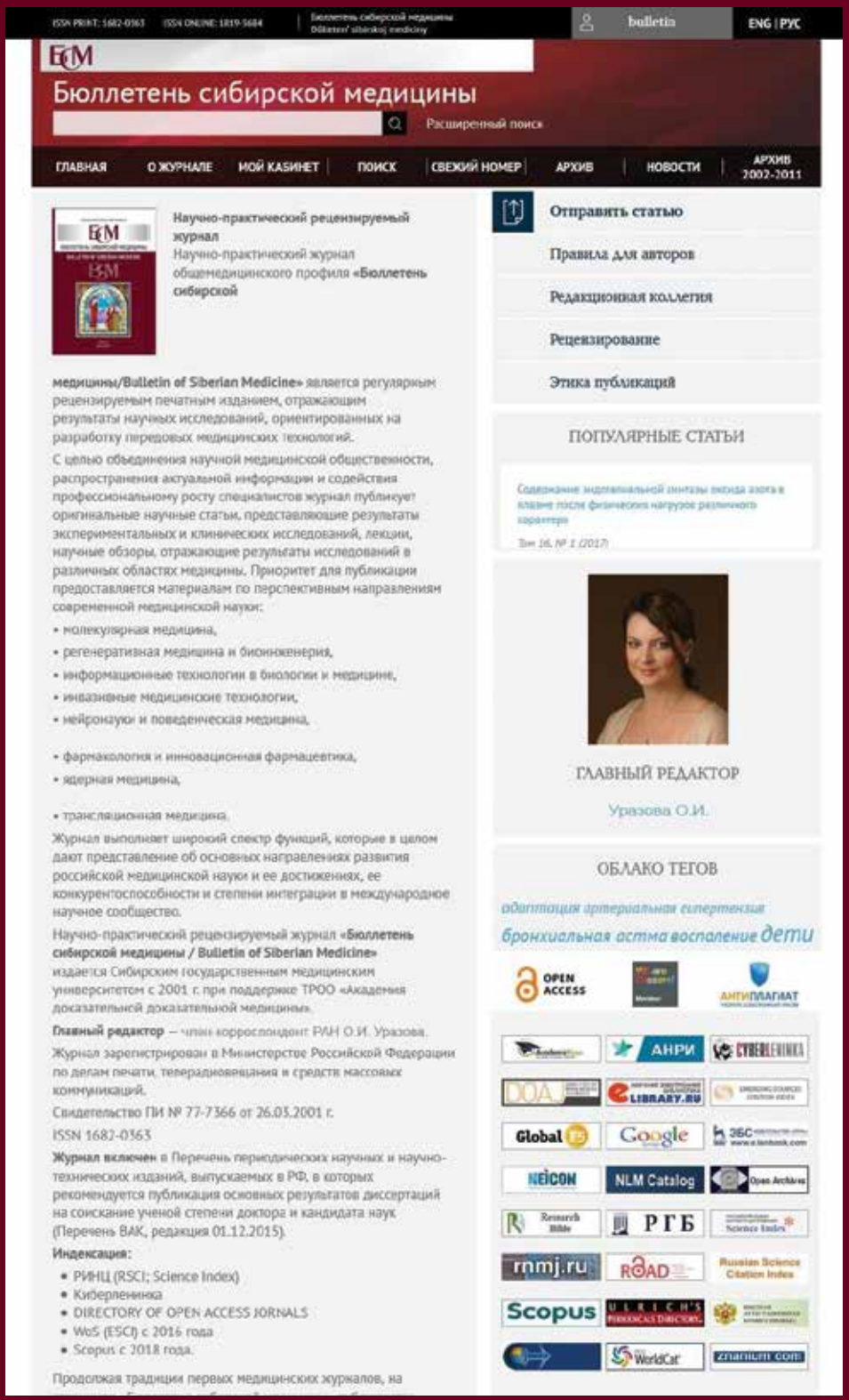

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