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# Features of the cytogram and cytokine profile of bronchoalveolar lavage fluid in experimental metabolic syndrome

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## ABSTRACT

The aim of the study was to identify the features of the cellular composition and cytokine profile of bronchoalveolar lavage fluid in rats in a model of diet-induced metabolic syndrome.

**Materials and methods**. In an experiment on animals (rats), a model of metabolic syndrome (MS) induced by a high-fat and high-carbohydrate diet was reproduced. To assess the viability of the reproduced model, biochemical and morphometric methods were used, such as measurement of body weight, specific gravity of liver and visceral fat, and blood pressure, determination of glucose concentration in the blood (including a glucose tolerance test), as well as determination of blood lipid parameters. To assess the intensity of the inflammatory response in the blood, the concentration of total protein, the total number of leukocytes, and the levels of immunocytokines (interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)α, monocyte chemoattractant protein (MCP)-1) were determined. Open bronchoalveolar lavage was performed on the isolated heart – lung complex. The concentration of protein, immunocytokines (IL-6, IL-10, TNFα, MCP-1), the total number of leukocytes, and the ratio of their morphological types were determined in the bronchoalveolar lavage fluid (BALF).

**Results.** In animals with MS, an increase in the total number of leukocytes in the blood due to granulocytes and a rise in the concentration of protein, TNFα, and IL-10 were revealed compared with the parameters in the controls. BALF analysis revealed an increase in the concentration of protein, the total number of leukocytes, and the absolute number of alveolar macrophages, neutrophil granulocytes, and lymphocytes. The levels of IL-6 and MCP-1 were more than 1.5 times higher.

**Conclusion.** Changes in the qualitative and quantitative parameters of BALF are inflammatory in nature and are formed during a systemic inflammatory response accompanying metabolic disorders in modeling MS in rats in the experiment.

**Keywords:** metabolic syndrome, bronchoalveolar lavage fluid, inflammation

**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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# Особенности цитограммы и цитокинового профиля жидкости бронхоальвеолярного лаважа при экспериментальном метаболическом синдроме

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**РЕЗЮМЕ**

**Цель.** Выявить особенности клеточного состава и цитокинового профиля жидкости бронхоальвеолярного лаважа у крыс в модели диет-индуцированного метаболического синдрома.

**Материалы и методы.** В эксперименте на животных (крысах) воспроизведена модель метаболического синдрома (МС), индуцированного высокожировой и высокоуглеводной диетой. Для оценки состоятельности воспроизведенной модели использованы биохимические и морфометрические методы: измерение массы тела, удельной массы печени и висцерального жира, измерение артериального давления, определение содержания в крови глюкозы (в том числе в глюкозотолерантном тесте (ГТТ)), определение параметров липидного спектра крови. Для оценки интенсивности воспалительного ответа в крови определяли концентрацию общего белка, общее количество лейкоцитов и концентрацию иммуноцитокинов (интерлейкина (IL) -6, IL-10, фактора некроза опухоли альфа (TNFα), моноцитарного хемотоксического фактора-1 (MCP-1)). Открытым способом на изолированном комплексе «сердце–легкие» выполняли бронхоальвеолярный лаваж. В бронхоальвеоларной жидкости (БАЛЖ) определяли концентрацию белка, иммуноцитокинов (IL-6, IL-10, TNFα, MCP-1), общее количество лейкоцитов и соотношение их отдельных морфологических форм.

**Результаты.** У животных с МС выявлено повышение в крови общего количества лейкоцитов за счет гранулоцитарного компонента, увеличение концентрации белка и цитокинов TNFα и IL-10 по сравнению с соответствующими параметрами у крыс контрольной группы. В результате анализа БАЛЖ выявлено повышение концентрации белка, общего количества лейкоцитов, абсолютного числа альвеолярных макрофагов, нейтрофильных гранулоцитов и лимфоцитов; более чем в 1,5 раза превышена концентрация IL-6 и MCP-1.

**Заключение.** Изменения качественных и количественных параметров БАЛЖ носят воспалительный характер и формируются на фоне системного воспалительного ответа, сопровождающего нарушение обмена веществ при моделировании МС у крыс в эксперименте.

**Ключевые слова:** метаболический синдром, бронхоальвеолярная жидкость, воспаление

**Конфликт интересов.** Авторы гарантируют отсутствие потенциальных и явных конфликтов интересов, связанных с публикацией данной статьи.

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## INTRODUCTION

A cluster of metabolic conditions united under the term metabolic syndrome (MS) accelerate the development and progression of a number of diseases, including cardiovascular and cerebrovascular diseases, type 2 diabetes mellitus, kidney and biliary tract diseases, and some types of cancer [1–3]. In the clinical medicine with wide-spread comorbidity, one of the discussed issues is the effect of the MS components on the state of the respiratory system. In some clinical studies, MS has been identified as an independent risk factor for impaired lung function and aggravation of respiratory symptoms in multifactorial

diseases, such as bronchial asthma and chronic obstructive pulmonary disease.

It has been established that the most significant MS components contributing to respiratory pathology are abdominal obesity, hyperglycemia, and hyperinsulinemia [4–6]. Early mechanisms of the damaging effect of the MS components on the bronchopulmonary system remain the least studied. To some extent, this is due to the fact that most studies are clinical in nature. Since the cardiorespiratory system has broad adaptive capabilities, patients with pronounced manifestations of respiratory failure against the background of MS are in the focus of attention of doctors. In this regard, it is advisable to study the complex mechanisms of the effect of metabolic disorders on the morphological and functional state of the bronchopulmonary system in an experiment using animal models.

Collecting and studying bronchoalveolar lavage fluid (BALF) are reliable methods for studying the cytological, immunological, biochemical, and microbiological characteristics of the bronchoalveolar parts of the respiratory system. The study of BALF not only provides significant assistance in diagnosing and determining the activity of the pathological process, but also allows for a deeper understanding of the pathogenetic patterns of lung damage in the underlying or concomitant pathology [7, 8].

The aim of the study was to reveal the features of the cellular composition and cytokine profile of BALF in rats in a model of diet-induced MS.

## MATERIALS AND METHODS

The experiment was performed on 33 outbred male Wistar rats (average weight 280.5 ± 36.1 g) aged 6 weeks at the beginning of the study, which were divided into control (15 animals) and experimental (18 animals) groups. The animals were kept in the conditions of a vivarium. The studies were carried out in compliance with the principles of humanity set out in the directives of the European Community (86/609/EEC) and the Declaration of Helsinki.

A model of diet-induced MS was reproduced in the animals of the experimental group. The rats were fed with a high-fat and high-carbohydrate diet containing standard feed (66%) with the addition of animal fat (17%), fructose (17%), and cholesterol (0.25%); drinking water was replaced with a 20% fructose solution (total calorie content of the daily diet was 440 kcal / 100 g). The rats of the control group received a standard diet (Delta Feeds, BioPro, Russian Federation, total calorie content 300 kcal / 100 g, proteins 24%, fats 6%, carbohydrates 44%) with free access to food and water.

To assess the viability of the reproduced MS model, body weight and blood pressure were measured in the animals at the beginning and at the end of the experiment (Systola, Neurobotics, Russian Federation). In the last week of the experiment, a glucose tolerance test (GTT) was performed: fasted rats (fasting for 12 hours) were intragastrically injected with a glucose solution at a dose of 2 g / kg (D-glucose, Sigma-Aldrich, USA). After 0, 15, 30, 60, 90, and 120 min, the blood glucose concentration was determined by the enzymatic colorimetric method using a reagent kit (Chronolab, Spain). 12 weeks after the start of the experiment, the animals were euthanized by CO2 asphyxia. Blood was taken from the heart of the animals to assess hematological parameters (vacutainer K2EDTA tubes) and obtain blood serum (vacutainer serum clot activator tubes). Hematological parameters were assessed on the automatic hematology analyzer (BC-2800 Vet, Mindray, China). Biochemical parameters were determined in the blood serum, including lipid indices (on the Architect c4000 Automatic Biochemistry Analyzer, Abbot, USA). The levels of immunocytokines (interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)α, monocyte chemoattractant protein (MCP)-1) were measured by the enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems kits, GmbH, Austria). The liver and visceral adipose tissues (mesenteric, epididymal, and retroperitoneal adipose tissue) were isolated by dissection and weighed on the analytical balance, and their specific gravity was calculated.

Open bronchoalveolar lavage was performed on the isolated heart – lung complex. Cold saline was used as a lung lavage fluid [9]. Both lungs were washed 2–3 times with a truncated syringe inserted via the trachea. The initial volume of the lavage fluid for a single injection was 3 ml, the return volume was at least 2 ml. In the BALF, the protein concentration was determined spectrophotometrically by the BCA assay (BCA Protein Assay Kit, Sigma-Aldrich), and cytokines IL-6, IL-10, TNFα, and MCP-1 were measured by ELISA (Bender MedSystems GmbH kits, Austria).

BALF cytology was performed to identify the total number of leukocytes and the ratio of their morphological types. For this purpose, BALF was centrifuged, and cell pellet sections were placed on a glass slide, fixed in formaldehyde vapor, and stained

using the Romanowsky – Giemsa stain. Cellular elements were counted per 200 cells using microscopy with immersion oil objective.

Statistical analysis was performed using the SPSS Statistics 23 software. Normally distributed data (Shapiro – Wilk test) were presented as the mean and the standard deviation ($M \pm SD$). Non-normally distributed data were presented as the median and the interquartile range Me ($Q_{25}$;$Q_{75}$). Differences between the samples were analyzed using the Student's *t*-test or the Mann – Whitney *U* test. The differences were considered statistically significant at $p < 0.05$.

## RESULTS

The impact of a high-fat and high-carbohydrate diet on the animals of the experimental group led to statistically significant changes in the physiological and biochemical parameters compared with the controls: an increase in the body weight, an increase in the specific gravity of the liver and visceral adipose tissue, a rise in the blood pressure, an increase in the concentration of total protein and glucose in the blood (Table 1). Changes in the blood lipid indices were characterized by an increase in the concentration of triacylglycerols, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and very-low-density lipoprotein cholesterol (VLDL-C). The concentration of high-density lipoprotein cholesterol (HDL-C) was lower than that in the controls, which was manifested through the atherogenic coefficient, which was more than 1.5 times ($p = 0.02$) higher than in the control group (Table 1).

Table 1

**The effect of a high-fat and high-carbohydrate diet on the physiological and biochemical parameters of rats, $M \pm SD$**

| Parameter | Control group ($n = 15$) | Model of MS ($n = 18$) |
|---|---|---|
| Body weight, g | 433.3 ± 39.4 | 489.1 ± 47.9; $p = 0.01$ |
| Systolic blood pressure, mm Hg | 130.4 ± 9.5 | 145.1 ± 8.7; $p = 0.01$ |
| Diastolic blood pressure, mm Hg | 86.5 ± 9.3 | 101.4 ± 12.2; $p = 0.028$ |
| Fasting blood glucose, mmol / l | 4.7 ± 0.5 | 6.6 ± 0.4; $p < 0.001$ |
| Total protein, g / l | 52.7 ± 3.4 | 66.7 ± 3.8; $p = 0.004$ |
| Total cholesterol, mmol / l | 1.7 ± 0.2 | 2.3 ± 0.3; $p = 0.001$ |
| HDL-C, mmol / l | 0.6 ± 0.1 | 0.4 ± 0.1; $p = 0.003$ |
| LDL-C, mmol / l | 0.9 ± 0.2 | 1.4 ± 0.4; $p = 0.02$ |
| VLDL-C, mmol / l | 0.3 ± 0.1 | 0.5 ± 0.1; $p = 0.03$ |
| Triacylglycerols, mmol / l | 0.7 ± 0.2 | 1.7 ± 0.5; $p = 0.001$ |
| Atherogenic coefficient | 2.5 ± 0.3 | 3.8 ± 0.7; $p = 0.02$ |
| Specific gravity of the adipose tissue, g | 2.2 ± 0.2 | 4.3 ± 0.6; $p < 0.001$ |
| Specific gravity of the liver, g | 3.1 ± 0.4 | 4.2 ± 0.5; $p < 0.001$ |

Table 1 (continued)

Note: here and in Tables 2–4: *p* is the level of statistical significance of the differences compared with the parameters in the control group.

In the animals with MS, GTT revealed a decrease in glucose tolerance. An increase in the area under the "glucose concentration – time" curve by 1.3 times was recorded on the graph showing changes in the blood glucose levels compared with values in intact animals ($AUC_{0\text{-}120}$). In the control group, the area under the curve (AUC) was 752.2 ± 50.4 mmol / l × 120 min, in the experimental group – 940.9 ± 55.8 mmol / l × 120 min ($p = 0.001$) (Figure).

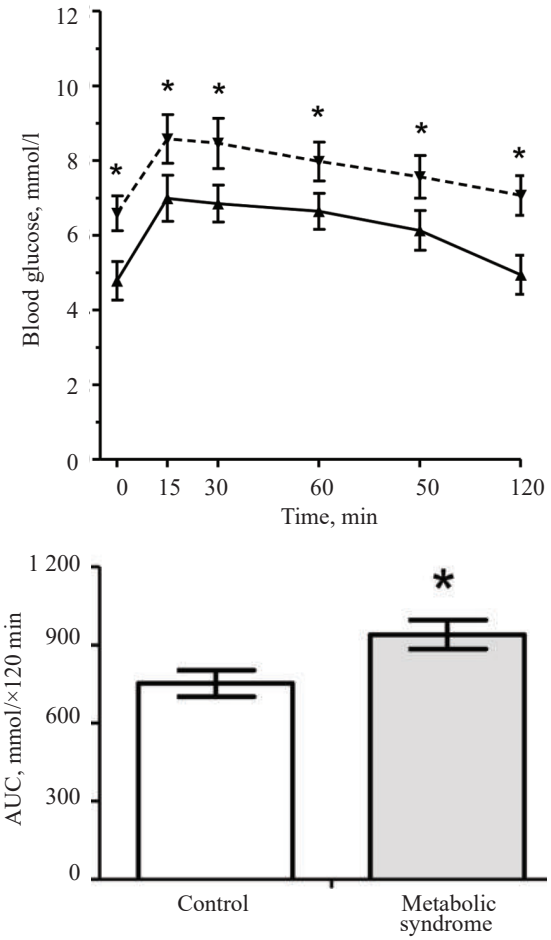


Figure. Changes in the concentration of glucose in the blood of rats (*a*) and the area under the "glucose concentration – time" curve ($AUC_{0\text{-}120}$) (*b*) in the glucose tolerance test: solid line – control group, dotted line – experimental group.

*$p < 0.05$ compared with the control group

The analysis of hematological parameters revealed an increase in the total number of leukocytes in the blood of the experimental group animals by 1.4 times compared with the values in the control group. The quantitative analysis of the leukocyte differential revealed a statistically significant increase in the absolute and relative number of granulocytes per unit volume of blood (Table 2).

According to the results of the biochemical analysis, the protein concentration in the BALF in the animals of the experimental group was 1.08 (±0.30) g / l, which, on average, was 1.5 times higher ($p = 0.037$) than the corresponding value in the intact animals (Table 3). The qualitative and quantitative analysis of the BALF cytogram revealed an increase in the total number of leukocytes per unit volume of fluid, compared with the control values, due to an increase in the absolute number of all types of leukocytes in the BALF – alveolar macrophages, neutrophil granulocytes, and lymphocytes (Table 3).

The study of the concentration of cytokines in the blood serum of the experimental animals revealed higher levels of IL-10 and TNFα compared with the control values. The concentration of IL-6 and MCP-1 in the BALF significantly exceeded the control values (Table 4).

Table 2

**Total number of leukocytes and hemogram parameters in the experimental animals, *Me* ($Q_{25}$; $Q_{75}$)**

| Parameter | | Control group ($n = 15$) | Model of MS ($n = 18$) |
|---|---|---|---|
| Total number of leukocytes, $\times 10^9$ / l | | 9.9 (9.4; 10.9) | 13.7 (11.4; 15.0); $p = 0.001$ |
| The quantitative composition of cells (in the numerator – in %, in the denominator – in absolute numbers, $\times 10^9$/l) | Granulocytes | 28.2 (25.9; 31.3)<br>2.5 (1.7; 3.6) | 33.2 (31.5; 34.2); $p = 0.001$<br>3.9 (3.2; 4.4); $p = 0.003$ |
| | Lymphocytes | 65.3 (64.2; 67.6)<br>7.6 (5.9; 8.3) | 64.2 (62.7; 66.2); $p = 0.343$<br>7.1 (6.4; 8.5); $p = 0.84$ |
| | Monocytes | 3.4 (3.0; 3.6)<br>0.4 (0.2; 0.4) | 3.5 (3.1; 4.0); $p = 0.1$<br>0.5 (0.3; 0.4) $p = 0.166$ |

Table 3

**Protein concentration, *M* ± *SD*, the total number of leukocytes, and the parameters of the BALF cytogram in the experimental animals, *Me* ($Q_{25}$; $Q_{75}$)**

| Parameter | | Control group ($n = 15$) | Model of MS ($n = 18$) |
|---|---|---|---|
| Total protein, g / l | | 0.74 (±0.20) | 1.08 (±0.30)<br>$p = 0.037$ |
| Total number of leukocytes, $\times 10^9$ / l | | 0.55 (0.30; 0.84) | 0.80 (0.65; 1.55)<br>$p = 0.047$ |
| Quantitative composition of cells (in the numerator – in %, in the denominator – in absolute numbers, $\times 10^9$ / l) | Alveolar macrophages | 42.60 (38.50; 53.00)<br>0.23 (0.13; 0.39) | 51.50 (33.88; 55.25)<br>$p = 0.677$<br>0.66 (0.51; 0.87)<br>$p = 0.0007$ |
| | Neutrophil granulocytes | 47.00 (41.60; 55.50)<br>0.27 (0.14; 0.36) | 45.25 (36.50; 55.50)<br>$p = 0.589$<br>0.58 (0.02; 0.09)<br>$p = 0.035$ |
| | Lymphocytes | 5.50 (1.75; 7.75)<br>0.02 (0.01; 0.04) | 7.00 (2.38; 9.88)<br>$p = 0.146$<br>0.05 (0.02; 0.09)<br>$p = 0.039$ |

Table 4

**Concentration of cytokines, pg / ml, in the blood serum and BALF of the experimental animals, *Me* ($Q_{25}$; $Q_{75}$)**

| Parameter | Blood serum | | BALF | |
|---|---|---|---|---|
| | Control group ($n = 15$) | Model of MS ($n = 18$) | Control group ($n = 15$) | Model of MS ($n = 18$) |
| IL-6 | 5.5 (2.3; 6.3) | 7.8 (4.7; 14.1); $p = 0.152$ | 5.3 (4.7; 9.2) | 9.7 (9.4; 15.7); $p = 0.007$ |
| IL-10 | 11.8 (6.0; 23.8) | 43.3 (21.9; 54.7); $p = 0.029$ | 59.9 (37.4; 74.5) | 66.1 (38.4; 85.9); $p = 0.351$ |
| TNFα | 2.6 (2.6; 5.2) | 10.8 (6.4; 11.7); $p = 0.035$ | 40.1 (20.6; 46.2) | 39.6 (31;5; 42.5); $p = 0.863$ |
| MCP-1 | 158.6 (91.7; 454.6) | 155.7 (111.7; 407.3); $p = 0.423$ | 166.7 (131.5; 352.5) | 284.3 (184.0; 498.1); $p = 0.045$ |

## DISCUSSION

Reproduction of genetic or diet- and drug-induced models of MS in experiments on animals makes it possible to study changes in homeostatic parameters and analyze the effect of emerging metabolic disorders on various organs and systems. Diet-induced combined *in vivo* models of MS with a high content of fat and carbohydrates in the diet are more similar to the unbalanced human diet, are most consistent with alimentary obesity, and are adequate in terms of the mechanisms of development of MS and associated comorbidity [10–12].

The model reproduced in our experiment reflected the main biometric and biochemical changes typical of MS. The rats receiving a 12-week high-fat and high-carbohydrate diet had an increase in the body weight due to the accumulation of visceral adipose tissue and hepatomegaly. Besides, arterial hypertension with an increase in both systolic and diastolic blood pressure was noted in the animals with MS (Table 1). Blood biochemistry revealed changes indicating carbohydrate and lipid metabolism disorders, such as fasting hyperglycemia, low glucose tolerance, as well as dyslipoproteinemia with an increase in the blood level of triacylglycerols and atherogenic fractions of lipoproteins (LDL-C, VLDL-C) and a decrease in the concentration of high-density lipoproteins.

A number of studies have confirmed that factors of the immune system are actively involved in the pathogenesis of digestive diseases [13, 14]. An important role in the pathogenesis of MS is attributed to sterile inflammation in the adipose tissue, which is induced by macro- and micronutrients, as well as metabolic products formed in the visceral adipose tissue with its excessive accumulation [15]. Such metabolic inflammation often does not have pronounced clinical manifestations, but is accompanied by local stromal vascular and functional changes in the adipose tissue – adipocyte hypertrophy, infiltration by immune cells, fibrosis of the extracellular matrix, impaired microcirculation, and changes in the secretory phenotype of cellular elements [14]. However, in some cases, laboratory tests reveal an increase in the level of nonspecific inflammatory markers in the blood, such as C-reactive protein, fibrinogen, procalcitonin, etc., which correlates with the severity of inflammation in the adipose tissue [16].

As a result of our experiment, we revealed an increase in the total number of leukocytes due to granulocytes, as well as an increase in the protein concentration in the blood serum of the animals with induced MS compared with the intact animals (Tables 1, 2). At the same time, we registered an increase in the levels of TNFα and IL-10 in the blood serum (Table 4). The identified changes are common signs of an inflammatory response that develops against the background of diet-induced metabolic disorders [17–19].

Factors of systemic inflammation associated with MS and obesity contribute to the development of pathology in various organs and systems, which is confirmed by a large number of experimental and clinical studies [2, 20]. One of the informative methods for detecting biological markers of most lung diseases is the study of BALF (cytological, biochemical, immunological), which gives accurate information about the direction and severity of protective, adaptive, and pathological reactions in the lungs and allows to study the systemic mechanisms for maintaining structural and functional homeostasis in the bronchopulmonary system [7, 8].

Following the analysis of the BALF cytogram, we found an increase in the total number of leukocytes, as well as their individual morphological types (the absolute number of alveolar macrophages, neutrophil granulocytes, and lymphocytes) in the animal models of MS compared with the control values (Table 3). In normal conditions, macrophages represent the majority of phagocytes in the lower respiratory tract [21]. During inflammation, the proportion of alveolar macrophages increases both due to proliferation of tissue-resident cells (to a larger extent) and due to recruitment of macrophages of monocytic origin from peripheral blood (to a lesser extent) [22]. Monocytes infiltrating tissues find themselves in a specific microenvironment and develop into macrophages with altered functions, often exacerbating inflammatory responses [23]. It is assumed that it is monocytes recruited from the blood, and not proliferating tissue-resident alveolar macrophages, that are the precursors of M1-polarized macrophages in the lungs. The latter are distinguished by pronounced cytotoxic and antimicrobial activity, secrete a large number of reactive oxygen species, nitrogen, and proinflammatory cytokines, and contribute to alteration and progression of inflammation in the bronchial mucosa [24].

The analysis of the BALF cytokine profile revealed a more than 1.5-fold increase in the concentration of IL-6 and MCP-1 in the animals with MS compared with the controls (Table 4). It is known that the monocyte chemotactic factor is predominantly secreted by macrophages and

monocytes, dendritic cells, and lung fibroblasts and increases the chemotactic activity of monocytes, but not neutrophils [25]. IL-6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties due to implementation of different types of signaling [26]. In chronic obstructive pulmonary disease, IL-6 is crucial for attracting neutrophils to the site of inflammation, as it leads to synthesis of the necessary chemokines by endothelial cells as a result of trans-signaling. At the same time, neutrophils attracted to the focus of inflammation are some of the sources of soluble IL-6 receptor (sIL-6R), which is necessary for initiating trans-signaling in relation to the structural cellular elements of the lungs (fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells) [27, 28]. In turn, excessive proinflammatory activity of the cellular components in the pulmonary interstitial matrix can lead to an increase in the permeability of the alveolar – capillary barrier, which is confirmed by the increase in the protein concentration in the BALF in the group of animals with MS (Table 3).

## CONCLUSION

Experimental diet-induced MS is accompanied by the development of a systemic inflammatory response, laboratory signs of which are neutrophilic leukocytosis in the blood and an increase in the serum concentration of protein, TNFα, and IL-10. Organ-specific changes that characterize the negative impact of MS factors on the state of the bronchopulmonary system in the experimental animals are qualitative and quantitative changes in the composition of BALF, such as an increase in the protein concentration, an increase in the content of cellular elements (alveolar macrophages, neutrophil granulocytes, lymphocytes), and a rise in the concentration of proinflammatory cytokines IL -6 and MCP-1. Since these changes are inflammatory in nature, it seems relevant to evaluate structural, morphological, and functional changes in the bronchoalveolar system in laboratory animals that occur against the background of MS and assess their intensity in conjunction with systemic and local inflammation factors.

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Voronkova O.V., Birulina J.G., Ivanov V.V.– conception and design, drafting of the manuscript. Buyko E.E., Grigorieva A.V., Chernyshov N.A., Motlokhova E.A. – carrying out of the experiment. Osikhov I.A., Esimova I.E. – analysis and interpretation of the data.

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