

EXPERIMENTAL RESEARCHES

COMPARATIVE ANALYSIS OF *TRPM8* GENE AND COPD MARKERS EXPRESSION IN RAT LUNGS FOLLOWING EXPOSURE TO CIGARETTE SMOKE AND TOBACCO AEROSOL

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RESUME

Background. Chronic Obstructive Pulmonary Disease (COPD) remains a global medical and social problem. The ion channel *TRPM8*, a cold receptor, is known to be involved in the pathogenesis of the disease. However, existing experimental COPD models often exclude verification of changes in its expression.

Objective. To model COPD-like changes in rats exposed to cigarette smoke and tobacco aerosol, with a comprehensive assessment of morphological changes in lung tissue and the expression level of the *TRPM8* gene, as well as key COPD marker genes.

Materials and methods. Male Wistar rats were divided into one control and two experimental groups ($n = 30$). For 42 days, the first experimental group was subjected to daily inhalation exposure to cigarette smoke (CS), and the second group to tobacco aerosol (TA). On day 42, histological analysis of the lungs and assessment of the expression of COPD marker genes (*CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *VWF*) and *TRPM8* were performed using RT-PCR.

Results. CS exposure caused significant morphological changes: sclerosis (73.3 % of animals), infiltrate (100 %), emphysema (56.7 %), and goblet cell hyperplasia (90 %) compared to control ($p < 0.001$). TA led to less pronounced changes, and emphysema was absent. In the CS group, a significant increase in expression was observed: *COL4A2* by 63.5-fold, *IL2RA* by 7.9-fold, *CCL13* by 4.7-fold, and *TRPM8* by 2.2-fold. In the TA group, expression also increased significantly: *COL4A2* by 9.6-fold, *IL2RA* by 3.0-fold, *CCL13* by 7.0-fold, and *TRPM8* by 2.6-fold. CS induced a stronger expression of *COL4A2* and *IL2RA* compared to TA.

Conclusion. The model with 42-day CS exposure most adequately reproduces the COPD-like phenotype. Importantly, both experimental models caused a significant increase in the transcription of the cold receptor gene *TRPM8*, confirming its role in the pathogenesis and revealing new targets for therapy.

Keywords: COPD, *TRPM8*, *CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *VWF*, differential gene expression

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ ЭКСПРЕССИИ ГЕНА *TRPM8* И МАРКЕРОВ ХОБЛ В ЛЕГКИХ КРЫС ПРИ ИНТОКСИКАЦИИ СИГАРЕТНЫМ ДЫМОМ И ТАБАЧНЫМ АЭРОЗОЛЕМ

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РЕЗЮМЕ

Обоснование. Хроническая обструктивная болезнь легких (ХОБЛ) остается глобальной медико-социальной проблемой. Известно, что ионный канал *TRPM8*, являющийся рецептором холода, вовлечен в патогенез заболевания. Однако существующие экспериментальные модели ХОБЛ часто не включают верификацию изменений его экспрессии.

Цель. Смоделировать ХОБЛ-подобные изменения у крыс при ингаляционном воздействии сигаретного дыма и табачного аэрозоля, комплексно оценить морфологические изменения в легочной ткани и уровень экспрессии гена *TRPM8*, а также ключевых генов-маркеров ХОБЛ.

Материалы и методы. Самцов крыс линии Wistar разделили на контрольную и две экспериментальные группы ($n = 30$). Ежедневно в течение 42 дней проводили ингаляционное воздействие: на первую группу — сигаретным дымом (СД), на вторую — табачным аэрозолем (ТА). На 42-й день выполняли гистологический анализ легких и оценку экспрессии маркерных генов ХОБЛ (*CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *VWF*) и *TRPM8* методом ОТ-ПЦР.

Результаты. Воздействие СД вызывало значительные морфологические изменения: склероз (73,3 % животных), инфильтрат (100 %), эмфизема (56,7 %) и гиперплазия бокаловидных клеток (90 %) против контроля ($p < 0,001$). ТА приводил к менее выраженным изменениям, а эмфизема отсутствовала. В группе СД отмечали достоверное повышение экспрессии: *COL4A2* в 63,5 раза, *IL2RA* в 7,9 раза, *CCL13* в 4,7 раза и *TRPM8* в 2,2 раза. В группе ТА экспрессия также значимо увеличилась: *COL4A2* в 9,6 раза, *IL2RA* в 3,0 раза, *CCL13* в 7,0 раза и *TRPM8* в 2,6 раза. СД вызывал более сильную экспрессию *COL4A2* и *IL2RA*, чем ТА.

Заключение. Модель с 42-дневным воздействием СД наиболее адекватно воспроизводит ХОБЛ-подобный фенотип. Важно, что обе использованные экспериментальные модели вызывают значимое увеличение транскрипции гена холодовых рецепторов *TRPM8*, что подтверждает его роль в патогенезе и открывает новые мишени для терапии.

Ключевые слова: ХОБЛ, *TRPM8*, *CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *VWF*, дифференциальная экспрессия генов

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INTRODUCTION

According to WHO data, as of 2019, chronic obstructive pulmonary disease (COPD) became the cause of 3.3 million deaths, ranking third in the causes of mortality. The main risk factors for this disease include genetic and epigenetic alterations, as well as exposure to environmental factors, in particular cigarette smoke [1]. COPD is characterized by significant heterogeneity, which manifests in a diversity of clinical symptoms and endotypes, complicating diagnosis and treatment. Currently, the molecular targets for COPD therapy include β 2-adrenergic receptors, M-cholinoreceptors, glucocorticoid receptors, cyclic nucleotide phosphodiesterases specific to cAMP, as well as interleukin-4, -13, -33 receptors and CFTR ion channels [2]. Traditional universal approaches to treatment do not take into account the individual characteristics of patients, which reduces its effectiveness [3].

Cold receptors TRPM8, belonging to the melastatin subfamily of transient receptor potential ion channels [4], may represent a promising alternative for both therapy and diagnosis of COPD, since they play a role in various pathological processes characteristic of this disease. The spectrum of activating temperatures for TRPM8 varies somewhat according to different authors, but on average is 8–28°C, with an activity optimum at 10°C. It is known that exposure to low temperatures on the respiratory tract can lead to damage to the respiratory epithelium, as well as TRPM8-mediated excessive release of inflammatory mediators and recruitment of inflammatory cells into the respiratory tract [5]. In addition, stimulation of TRPM8 can cause increased production of mucin MUC5AC through a signaling cascade including an increase in intracellular Ca^{2+} concentrations, activation of phospholipase C (PLC), which carries out the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG), activating protein kinase C (PKC). A decrease in PIP2 and activation of PKC lead to the dissociation of the myristoylated alanine-rich C-kinase substrate (MARCKS) from the cell membrane, which weakens the epithelial barrier and facilitates the exocytosis of vesicles with mucin [6].

It is noteworthy that TRPM8 can also be activated under the action of dust particles and cigarette smoke – exogenous etiological factors of COPD. In this process, the main role, apparently, is played by reactive oxygen species initially contained in the smoke and secondarily formed in cells upon its action [5, 7].

Experimental blocking of TRPM8 significantly reduced the number of cells and the concentration of MIP-2 in the bronchoalveolar lavage, the expression of phosphorylated forms of kinases regulated by the extracellular signal (ERK), and N-terminal kinases of the transcription factor c-Jun (JNK), as well as the levels of inflammatory infiltration and damage in the lung tissues of mice against the background of 7-day exposure to cigarette smoke. Moreover, all

the listed changes were more pronounced when exposed to cigarettes with menthol (a TRPM8 agonist) compared with conditions in which smoke from regular cigarettes was used [8].

Apparently, cigarette smoke can increase the expression of TRPM8 in the respiratory tract, which was recorded in experiments in mice with subchronic exposure (1.5 cigarettes per day, 7 days) [8], as well as in rats with intensive exposure (10 cigarettes per hour, 6 hours a day, 40 days) [9]. Increased expression of TRPM8 mRNA and protein was also recorded in the bronchial epithelium of patients with COPD compared with healthy individuals [6].

As is known, one of the approaches to studying the mechanisms of disease development is the modeling of the disease in laboratory animals through prolonged exposure of cigarette smoke to the respiratory organs [10]. Such models allow reproducing COPD-like pathological processes in the lung tissue of animals, which allows studying the features of the pathogenesis of COPD under controlled conditions, including conducting an analysis of the differential expression of genes [11–13]. In this case, the effectiveness of modeling the pathology can be assessed not only by morphological criteria but also by specific changes in the expression of marker genes in the lung parenchyma. The following key marker genes can be used as such: the C-C chemokine receptor type 3 gene (*CCR3*), the C-C chemokine ligand type 13 gene (*CCL13*), the α 2-chain of type IV collagen (*COL4A2*), the α -subunit of the interleukin 2 receptor (*IL2RA*), and the von Willebrand factor (*VWF*), the differential expression of which was previously detected in patients with COPD using transcriptomic sequencing and subsequently confirmed in a rat model of the disease by real-time PCR with reverse transcription [14].

Taking into account the absence of existing COPD models with documented increased expression of TRPM8, the aim of the study was to model COPD-like changes in the respiratory system of rats under exposure to cigarette smoke or tobacco aerosol with verification of specific molecular and morphological biomarkers of the disease, as well as the level of TRPM8 gene expression in each of the models.

MATERIALS AND METHODS

The experiment was conducted on the basis of the Amur State Medical Academy in compliance with the ethical principles of conducting scientific medical research (excerpt from protocol No. 2 dated June 26, 2023). Exposure of animals to smoke and aerosol was carried out in a sealed plastic chamber with a volume of 9 liters, equipped with two ventilation openings 1 cm in diameter each, located on a removable lid to ensure air exchange and prevent excessive pressure.

Three groups of rats participated in the experiment: control, first experimental, and second experimental,

each group consisting of 30 animals. The control group was not exposed. Animals from the first experimental group received cigarette smoke (filter cigarettes “Maksim krasny”, Imperial Tobacco LLC, nicotine – 0.7 mg/cig), and in the second experimental group, exposure to tobacco aerosol was performed (nicotine – 0.7 mg/stick peo™, tobacco heating system glo™, ITMS Group of Companies).

Smoke/aerosol was obtained from one cigarette or one stick using a self-made syringe pump. The puff volume was 35 mL, duration – 2 s. After each puff, the cigarette was extinguished, and the stick was held for cooling of the heating element. The obtained smoke/aerosol was introduced into the exposure chamber. The procedure was repeated with an interval of 3 minutes (12 puffs) for the cigarette and 2 minutes 50 seconds (14 puffs) for the stick, which ensured complete use of one product with the same total exposure time of 40 minutes for all animals.

Heating tobacco to 350°C in special systems does not lead to the formation of new compounds that were not present in cigarette smoke when burning the same tobacco. The chemical differences between aerosol and smoke are mainly quantitative rather than qualitative: the aerosol contains the same set of compounds, but in significantly smaller amounts, with the exception of a small group of substances whose elevated level in tobacco aerosol is explained mainly by the presence of glycerol in the formulation [15].

Each rat was daily exposed to smoke from one cigarette or one stick for 40 minutes. On day 42, the animals were withdrawn from the experiment by cervical dislocation under chloroform anesthesia.

The choice of the 42-day period of intoxication of rats is based on the need to model stable pathophysiological changes characteristic of the early stages of COPD in humans. This period is minimally sufficient for the development of pronounced inflammation, oxidative stress, and the onset of airway remodeling [16].

The smoking dose (1 cigarette per day) was determined as a result of previous pilot observations, which established that a higher dose (≥ 2 cigarettes per day) under the tested conditions led to frequent premature mortality in animals. We did not use lipopolysaccharides and infectious pathogens in modeling COPD in order to achieve etiological relevance and recreate the natural course of the pathology without significant distortion of the inflammatory response [17].

The lungs were removed for histological evaluation and analysis of the expression levels of *CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *VWF*, and *TRPM8*. Lung tissues were fixed in 10 % formalin, dehydrated in alcohols of increasing concentration, and embedded in paraffin. Staining was performed with hematoxylin and eosin. The obtained preparations were examined using microscopy. During microscopy of sections stained with hematoxylin and eosin, characteristic histopathological markers of chronic inflammation of lung tissue were assessed: inflammatory infiltration (lymphocytes, neutrophils,

macrophages) peribronchially and perivascularly, airway remodeling (thickening of bronchial walls, goblet cell hyperplasia), destruction of alveolar septa (emphysema), and the development of interstitial fibrosis. Statistical analysis of the frequency of morphological changes was performed in the R environment (version 4.4.2; R Foundation for Statistical Computing). To identify general differences in the frequency of occurrence of morphological changes between the three experimental groups, the Fisher-Freeman-Halton exact test was used (the `fisher.test` function from the `stats` package), which is the preferred method for 2×3 contingency tables in the presence of zero or small expected frequencies. In the case of detecting statistically significant differences ($p < 0.05$), pairwise post hoc comparisons were performed using Fisher’s exact test for 2×2 tables with Bonferroni correction for multiple comparisons.

Total RNA was isolated from the remaining lung tissues using the RNA Solo kit (Evrogen, Russia). The concentration of total RNA after DNase treatment was measured on a NanoDrop spectrophotometer (ThermoFisher Scientific, USA). Assessment of the quality of the isolated RNA was performed using gel electrophoresis in a 2 % agarose gel, visualizing the 28S and 18S rRNA bands using the Gel Doc™ EZ Imager gel documentation system (Bio-Rad, USA). Quality samples without signs of RNA degradation had clearly pronounced intact 28S and 18S rRNA bands and a ratio of their intensities close to 2:1. cDNA was obtained from the isolated RNA by reverse transcription (RT) using the OT-MULV kit (Biolabmix, Russia). Subsequently, quantitative PCR was performed for all genes using the HS-qPCR SYBR-BLUE kit (Biolabmix, Russia). As a result of reverse transcription with oligo(dT) primers, a sufficient amount of cDNA was obtained for *CCR3*, *CCL13*, and *18S*, and for the genes *COL4A2*, *IL2RA*, *TRPM8*, *VWF*, gene-specific RT was performed. The 18S locus served as a constitutive (reference) gene, as the most stable in inflammatory processes in the lungs [14], and was subsequently used to calculate normalized expression values of the genes of interest. Real-time PCR included analysis of the melting curve after completion of the reaction (Fig. 1). Analysis of melting peaks was performed to confirm the specificity of amplification and the absence of nonspecific products or primer dimers. Amplification of six target genes was performed in separate tubes. After completion of the elongation stage, the amplifier recorded the fluorescence intensity with subsequent construction of derivative melting curves. The presence of a single narrow peak on each derivative curve for all six analyses indicated amplification of a specific product and the absence of side PCR products.

The presence of a single peak for each of the six genes indicates that the primers predominantly amplify the target DNA region.

The obtained samples were analyzed in four replicates on a CFX96 amplifier (BioRad, USA), using

the oligonucleotides specified in Table 1 [14]. As noted earlier, the marker genes *CCR3*, *CCL13*, *VWF*, *COL4A2*, and *IL2RA* were selected based on the results of a study in which differential analysis of the expression of various genes in lung tissue associated with the development of a COPD-like state was performed.

Amplification was performed under the following temperature conditions: initial denaturation – at 96°C for 2 min, then 32 cycles at a temperature-time regime: denaturation – at 94°C – 30 s, primer annealing at 60°C – 40 s, elongation – at 70°C for 1 min; final elongation – at 70°C for 2 min.

Statistical calculations of the relative expression of target genes by the $2^{-\Delta\Delta C_q}$ method were performed using the qRAT web service [18], with its plugin for ddCq analysis [19, 20].

RESULTS AND DISCUSSION

The most obvious morphological changes in the lungs were detected in animals receiving cigarette smoke. Thus, goblet cell hyperplasia was established, which, apparently, is a consequence of neutrophil infiltration during degranulation of which neutrophil elastase and cathepsin G are released (Fig. 2D) [21]. In the muscular membrane of the submucosal layer of the bronchi, hypertrophy and hyperplasia of myocytes were observed, which was due to a decrease in the number of glycogen granules in the muscle cells of the bronchi (Fig. 3A, 3D) [22]. On the part of the vessels, significant changes were noted, manifesting in the proliferation of intima and fibroelastosis, leading to a sharp narrowing of the lumen (Fig. 3C).

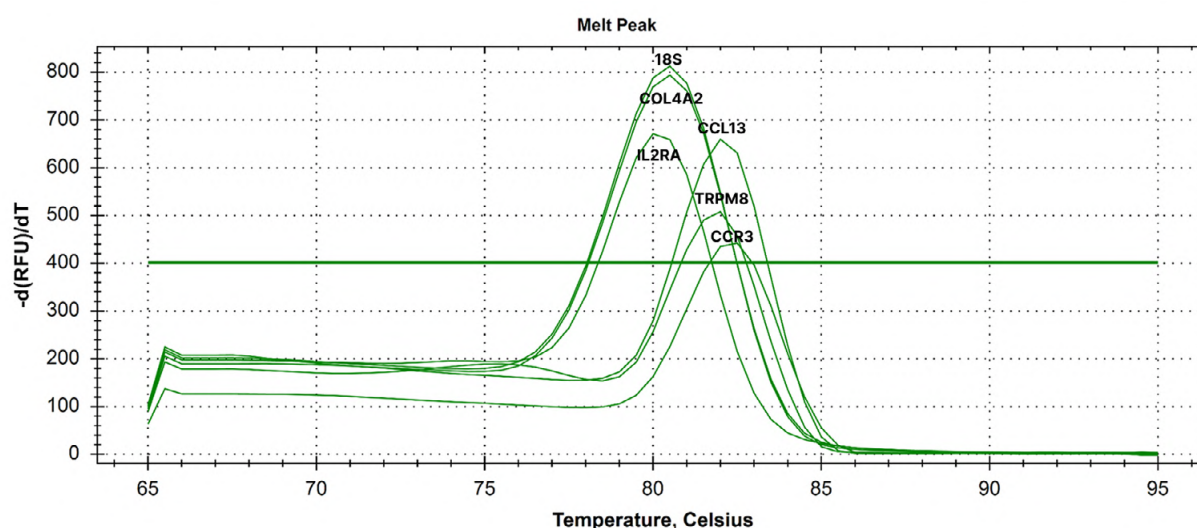


FIG. 1.

Melting peaks for *CCR3*, *CCL13*, *COL4A2*, *IL2RA*, *TRPM8*, *18S* gene products (compilation of six graphs)

TABLE 1
PRIMER SEQUENCES

Locus	Forward primer	Reverse primer	Amplicon length
<i>CCR3</i>	CCTGCTGACAATCGACAGGTA	GCTGCCAATACTGCAAGACC	120
<i>CCL13</i>	TCAGTGTTACCCCAGTCAC	GGAACTGGCTGCTTGTGAT	101
<i>VWF</i>	CCTTGGAAGTGGCTCGTCT	GCAAGTGCAGTTGACCAGG	88
<i>COL4A2</i>	CGAGAGGCGTCTCTGGATTC	TGCGTAAGGTTGCGCTTTCT	200
<i>IL2RA</i>	CCATAGTACCCGGCTGTTGG	CCGTTCTGTAGGAGAGGGC	91
<i>TRPM8</i>	GCAGTGGTACATGAACGGAGT	TGAAGAGTGAAGCCGAATAC	109
<i>18S</i>	CTTAGAGGGACAAGTGCGC	GGACATCTAAGGGCATCACA	70

Proliferation of the vascular intima in this case was due to hyperplasia of smooth muscle cells and accumulation of elastic and collagen fibers, which occurs as a result of damage to the endothelium of the intima, increased pressure in the system of the left gastric artery

and hypoxia [23] (Fig. 3C). Thickening of the bronchus walls (Fig. 2D, 3A, 3D) is associated with several pathological processes, including inflammation, hypertrophy of the mucous glands, fibrosis, and edema. Desquamation of the epithelium (Fig. 3B) and squamous

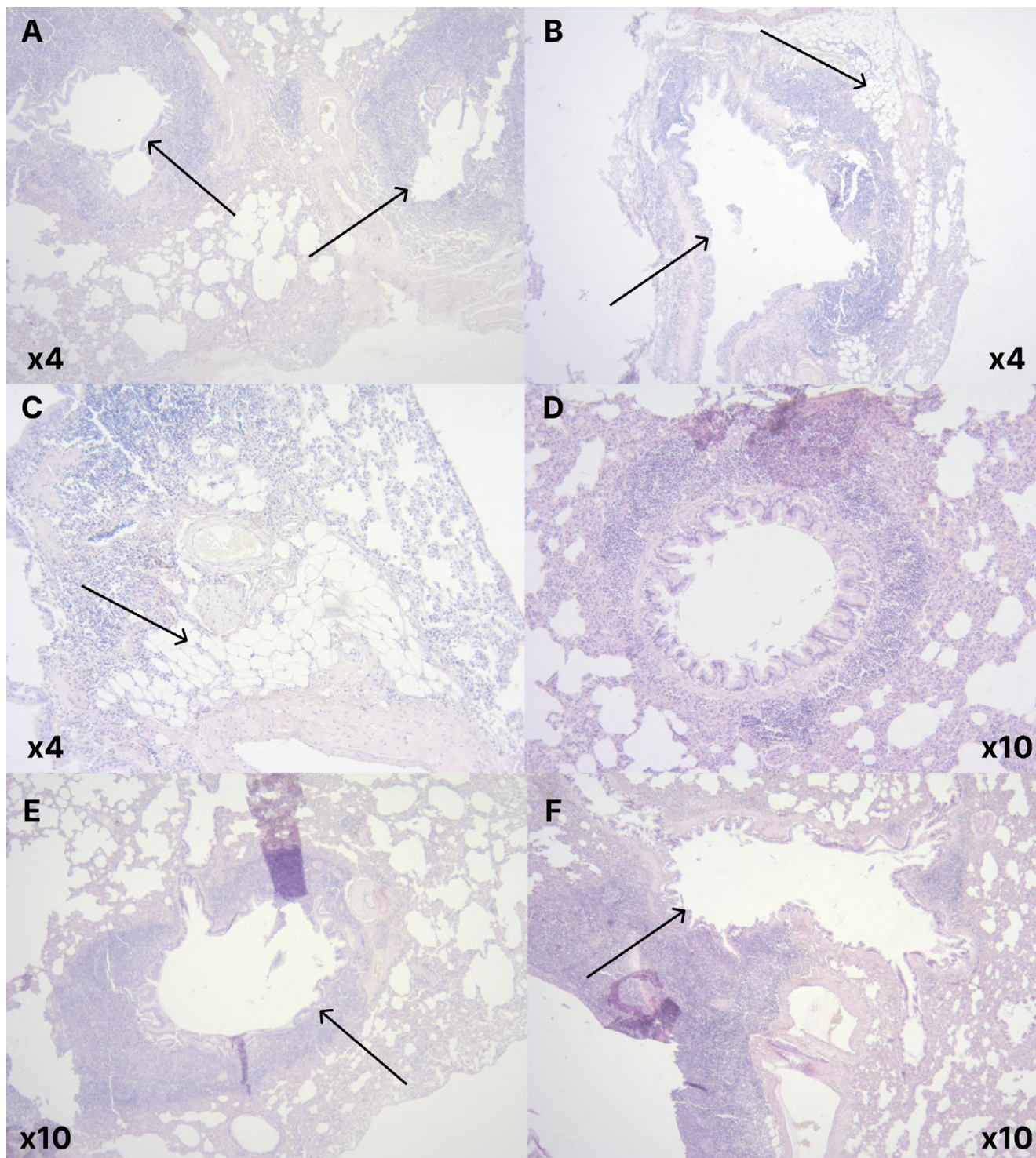


FIG. 2.

Histological images of the lungs of a rat chronically exposed to cigarette smoke (light microscopy, magnification x4 and x10, stained with hematoxylin and eosin). All images show serous-leukocytic infiltrate in the alveoli, sclerosis of the lung tissue, plethora and narrowing of the vascular lumen; A, B, E, F – bronchiectasis; B, C – foci of emphysematous changes in the lung tissue with bronchiectasis, areas of alveolar overstretching; D – thickening of the bronchial walls.

metaplasia (Fig. 3A) are the result of damage caused by chronic exposure to cigarette smoke and activation of reparative processes. Hyperplasia and hypertrophy of the glands, sclerosis of lung tissue were observed (Fig. 2A, 2E, 3A, 3D). Emphysematous change of lung tissue occurred, presumably induced by neutrophil proteases, including neutrophil elastase, proteinase 3, and cathepsin G, leading to damage to surrounding tissues. In addition, macrophage elastase, the activity of which increases against the background of smoking, may play a role in the development of emphysema, while its effect is not suppressed by $\alpha 1$ -antitrypsin, which allows this enzyme to destroy antiproteases (Fig. 2B and 2C) [24]. Against the background of exposure to tobacco aerosol, the changes in the lungs were less pronounced: infiltration of inflammatory cells, plethora, sclerosis of vessels, and remodeling of lung tissue were observed (Fig. 5A, 5B, 5C). Pathological changes (sclerosis of lung tissue, emphysematous

change, goblet cell hyperplasia) in the lungs of rats from the intact group were not observed (Fig. 4A, 4B).

Exposure to cigarette smoke causes the most pronounced pathological changes in lung tissue (sclerosis, infiltrate, emphysema, hyperplasia) compared with the intact group ($p < 0.001$). Tobacco aerosol also leads to significant changes, but to a lesser extent: it causes sclerosis and infiltrate, but not emphysema, and its effect is statistically weaker than that of smoke ($p < 0.001$ in pairwise comparisons). Thus, traditional smoking has a more destructive effect on the lungs than the use of tobacco aerosol (Table 2).

It was not possible to determine the expression of VWF, probably due to the very low concentration of transcripts of this gene in the studied sample. Despite the performance of gene-specific reverse transcription aimed at increasing the specificity of the reaction, the result remained negative. The obtained results of the analysis of the relative expression level of other

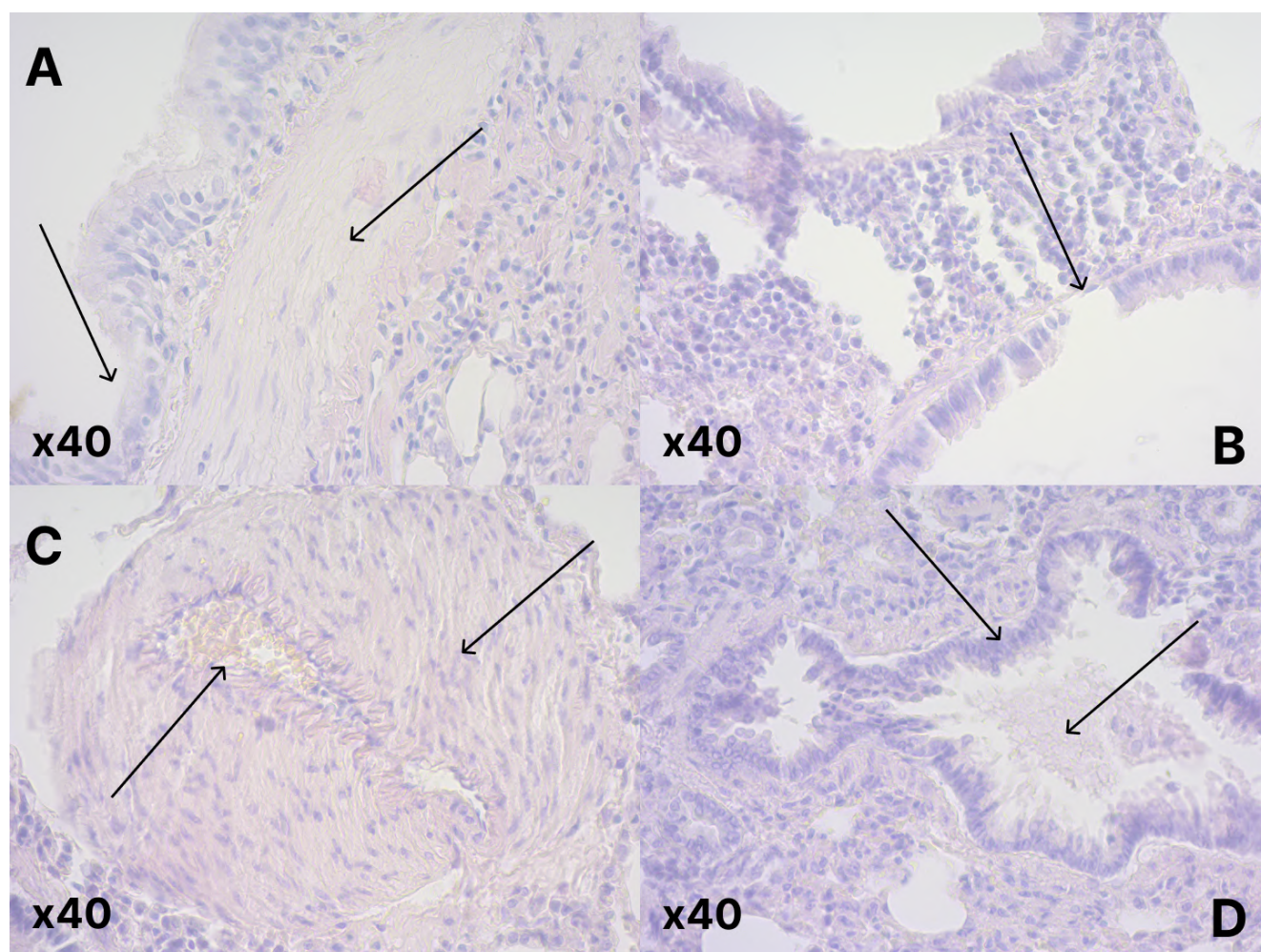


FIG. 3.

Histological images of the lungs of a rat chronically exposed to cigarette smoke (light microscopy, 40x magnification, hematoxylin and eosin staining). A – squamous cell metaplasia of the bronchial epithelium, hypertrophy and hyperplasia of myocytes; B – desquamation of the ciliated epithelium; C – plethora, sclerosis and fibroelastosis of the vessel, increased infiltration of perivascular tissue; D – goblet cell hyperplasia, leukocyte infiltrate in the bronchial lumen.

TABLE 2
PREVALENCE OF MORPHOLOGICAL CHANGES IN LABORATORY ANIMALS

Morphological change	Groups (n = 30)	% (n) with change	p-value (Fisher-Freeman-Halton test)	Pairwise comparisons (p-value with Bonferroni correction)
1. Sclerosis of lung tissue	Intact	0 (0)	< 0.001	$p_1 < 0.001$
	Cigarette smoke	73.3 (22)		$p_2 = 0.024$ (not significant)
	Tobacco aerosol	20.0 (6)		$p_3 < 0.001$
2. Serous-leukocytic infiltrate	Intact	10.0 (3)	< 0.001	$p_1 < 0.001$
	Cigarette smoke	100 (30)		$p_2 < 0.001$
	Tobacco aerosol	70.0 (21)		$p_3 < 0.001$
3. Emphysematous change	Intact	0 (0)	< 0.001	$p_1 < 0.001$
	Cigarette smoke	56.7 (17)		$p_2 = 1.0$ (not significant)
	Tobacco aerosol	0 (0)		$p_3 < 0.001$
4. Goblet cell hyperplasia	Intact	0 (0)	< 0.001	$p_1 < 0.001$
	Cigarette smoke	90.0 (27)		$p_2 < 0.001$
	Tobacco aerosol	30.0 (12)		$p_3 < 0.001$

Notes. *Intact vs. Smoke (p_1), Intact vs. Aerosol (p_2), Smoke vs. Aerosol (p_3).

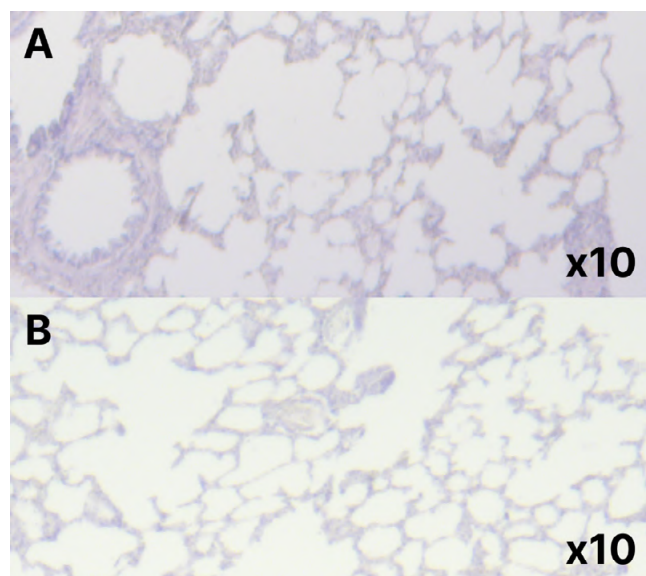


FIG. 4.
Histological images of the lungs of rats from the intact group (light microscopy, 10x magnification, hematoxylin and eosin staining). A, B – lungs without pathological changes.

genes (with the exception of *CCR3*) demonstrated an increase in their transcription in both experimental groups relative to the control (Fig. 6).

When comparing the experimental groups relative to each other, the absence of statistically significant differences was observed for most genes, with the exception of *COL4A2* and *IL2RA*, which were expressed

to a greater extent in the group exposed to cigarette smoke (by 1.7 ($p < 0.001$) and 1.5 times ($p = 0.04$)). Such differences indicate a more pronounced effect of cigarette smoke, compared with tobacco aerosol, on the proinflammatory reaction mediated through *IL-2RA* and increased transcription of *COL4A2*, which is responsible for the formation of collagen (Fig. 6).

The *CCR3* locus in both experimental groups had an insignificant change (Fig. 6) in expression. It is known that the *CCR3* receptor plays an important role in the development of allergic reactions and bronchial asthma, showing active expression in basophils, eosinophils, as well as in Th2 lymphocytes and epithelial cells lining the respiratory tract. Thus, its low expression indicates the absence of allergic inflammation in the respiratory tract of rats with a COPD-like state, which, despite the contradiction with the data of Guan Q., et al. [14], is generally characteristic of COPD [25].

CCL13 and *IL2RA*, like *CCR3*, are involved in various immune mechanisms associated with inflammation. *CCL13*, also known as MCP-4 (monocyte chemoattractant protein-4), is a CC chemokine possessing significant chemoattractant activity against monocytes, eosinophils, and basophils. This chemokine is significantly associated with allergic diseases such as bronchial asthma, but may not play a role in COPD [26]. Production of *CCL13* can be induced by inflammatory cytokines, including interleukin-1 and tumor necrosis factor alpha (TNF- α) [27]. The obtained data demonstrate statistically significant differences in the effect of tobacco aerosol and cigarette smoke

on the expression of the *CCL13* chemokine, which confirms the reliability of the observed effects. More pronounced expression of *CCL13* was observed under the action of tobacco aerosol, a 7.0-fold increase ($p < 0.05$), while under the action of cigarette smoke, a 4.7-fold increase ($p < 0.001$) was observed.

At the same time, for *IL2RA*, an opposite picture was observed: the expression of this gene increased by 7.9 times in the group receiving cigarette smoke ($p < 0.001$) (Fig. 6), and by 3.0 times ($p < 0.001$) in the group receiving tobacco aerosol (Fig. 6). The alpha chain of the interleukin-2 receptor (*IL2RA* or CD25)

plays a key role in the pathogenesis of autoimmune and oncological diseases, and increased expression of this protein is a marker of T-lymphocyte activation. CD25 can also be detected in non-lymphoid tissues, such as alveolar macrophages, Kupffer cells, and Langerhans cells. The role of this receptor in the development of COPD remains to be elucidated, however, there are studies demonstrating an increased content of soluble interleukin-2 receptor (sIL-2R) in the serum of smokers. The increased expression of sIL-2R induced by cigarette smoke plays a central role in the remodeling of the respiratory tract and damage to lung tissue.

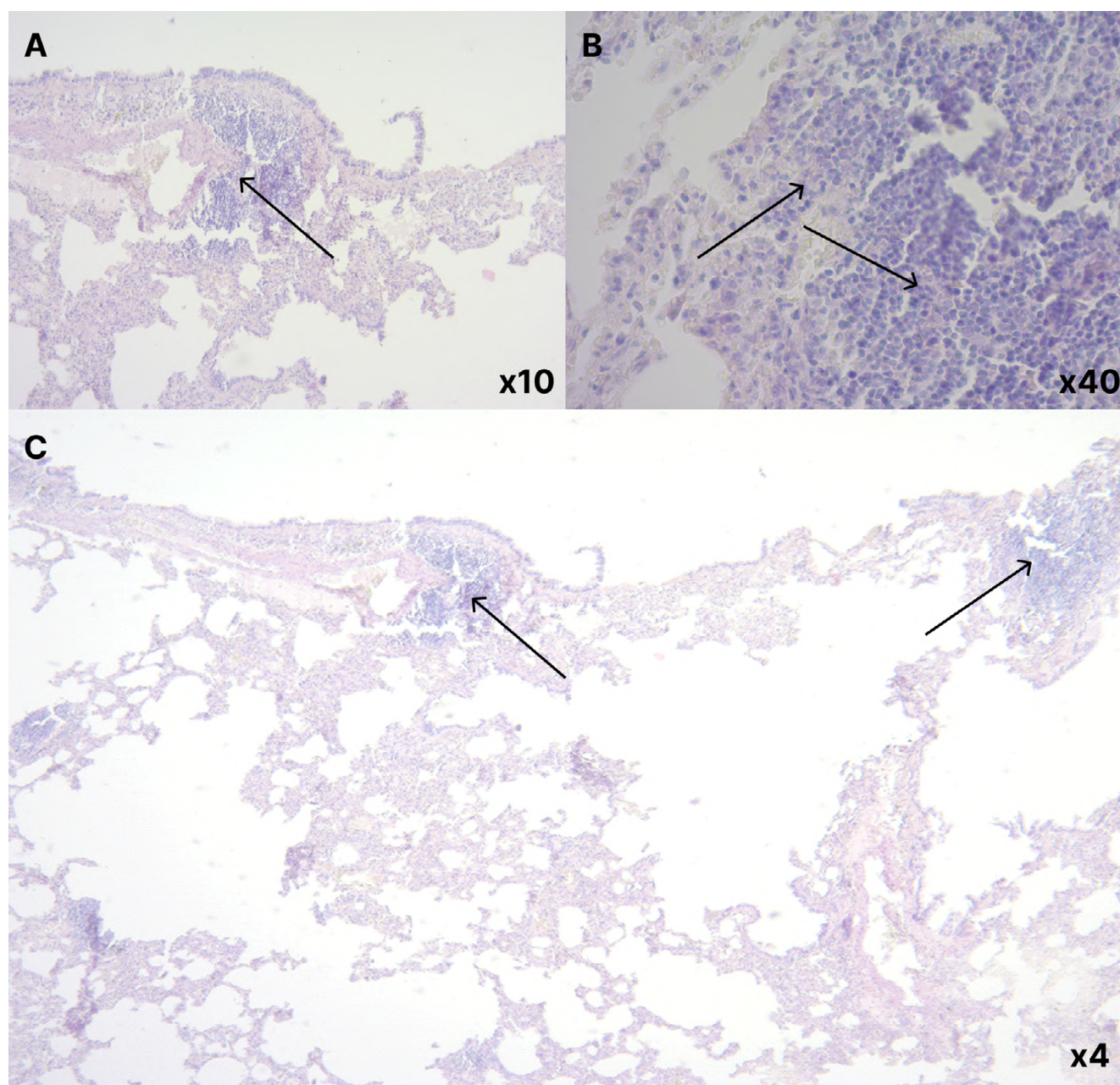


FIG. 5.

Histological images of the lungs of a rat chronically exposed to tobacco aerosol (light microscopy, magnification x4, x10, x40, stained with hematoxylin and eosin). A, B, C – lymphoid infiltration, A – perivascular infiltration, thickening of the vascular intima, sclerosis and vascular congestion.

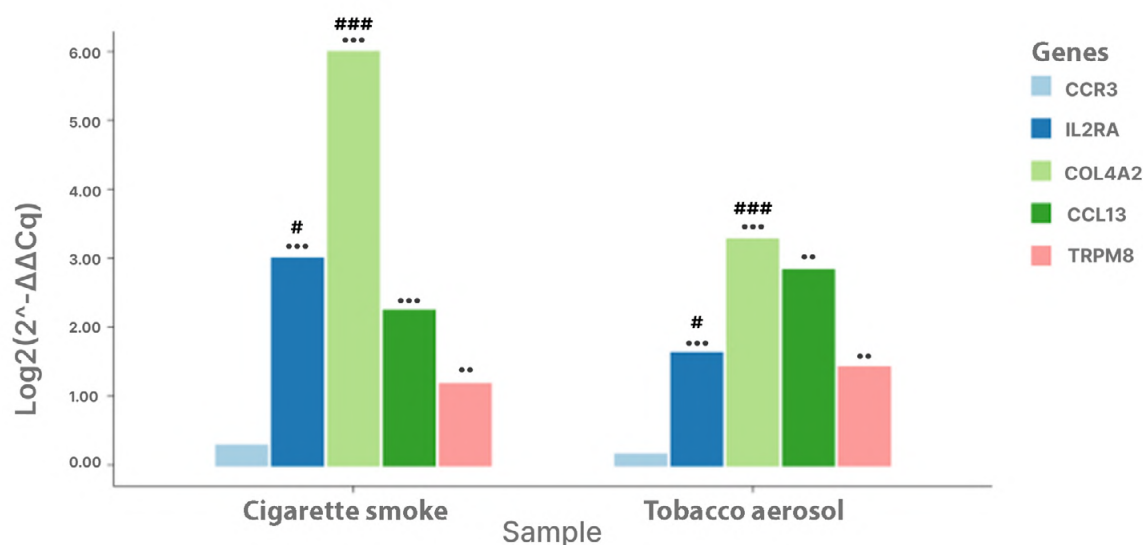


FIG. 6.

Graph of gene expression analysis in experimental groups versus control (on the vertical axis $\text{Log}_2(2^{-\Delta\Delta Cq})$).

Statistically significant differences *** $p < 0.001$ ** $p < 0.05$ – in relation to control; ### $p < 0.001$, # $p < 0.05$ – in relation of experimental groups to each other.

Studies show that even after cessation of smoking, inflammatory processes in the lungs continue to persist, which indicates the possible presence of autoimmune factors in chronic obstructive pulmonary disease (COPD). This allows us to suggest that the increased concentration of sIL-2R in the serum of smokers may indicate that cigarette smoke contributes to the occurrence and progression of COPD through the activation of T cells [28].

A significant increase in the expression of *COL4A2* was observed in both experimental groups: in the group receiving cigarette smoke, it was 63.5 times ($p < 0.001$) (Fig. 6), and in the group receiving tobacco aerosol – 9.6 times ($p < 0.001$) compared with the control group (Fig. 6). This is explained by several pathophysiological processes, among which chronic inflammation and damage to lung tissue are especially significant. In response to inflammatory signals, collagen synthesis increases in attempts to restore damaged areas [29]. It is known that COPD can be accompanied by the development of fibrosis, in which *COL4A2* performs a critical role as the main component of the extracellular matrix. Disruption of the structure of the extracellular matrix can lead to excessive collagen deposition in tissues and thickening of basement membranes. Additionally, hypoxia, which is often observed in COPD, can activate signaling pathways responsible for collagen synthesis, through the activation of hypoxia-inducible transcription factors. Based on the obtained data, it can be concluded that cigarette smoke had a greater effect on collagen expression compared with tobacco aerosol.

An increase in the synthesis of *TRPM8* mRNA compared with the control was comparable between

the group receiving cigarette smoke (2.2 times, $p = 0.006$) (Fig. 6) and the group receiving tobacco aerosol (2.6 times, $p = 0.002$) (Fig. 6). It is known that cold air, menthol, and solid particles contained in cigarette smoke can initiate inflammatory reactions through the *TRPM8*-mediated signaling pathway PKC/NF- κ B in primary epithelial cells of the respiratory tract. In this case, the result of activation of *TRPM8* is increased expression of many cytokines and chemokines, such as IL-1, IL-4, IL-6, IL-8, IL-13, granulocyte-macrophage colony-stimulating factor, and TNF- α [30].

Studies devoted to polymorphisms of the *TRPM8* gene also confirm its significant role in the pathogenesis of COPD. In particular, the *TRPM8* polymorphism rs2052030 may influence predisposition to the development of COPD, and carriers of the C allele of the rs11562975 polymorphism are predisposed to the formation of progressive bronchial obstruction [31, 32].

The main limitations of this study include the fact that the expression of COPD markers and *TRPM8* was assessed only at the mRNA level, which cannot reflect the expression of the corresponding proteins due to post-transcriptional regulation. In the study, we did not measure the number of leukocytes and inflammatory markers in the bronchoalveolar lavage, and did not evaluate the systemic level of inflammation. In addition, for the obtained histological preparations, we did not perform a quantitative morphometric analysis. Nevertheless, the changes in mRNA expression detected at the molecular level, as well as the morphological features of lung tissue after exposure to cigarette smoke indicate the formation of a pathological phenotype relevant to the COPD model.

CONCLUSION

In the present study, we tested the modeling of a COPD-like state in rats using inhalation of tobacco cigarettes and tobacco aerosol generated by tobacco heating systems. Despite the fact that the creation of a COPD model in animals using cigarette smoke often also includes inhalation of lipopolysaccharides [33], we chose a disease model that develops only under the influence of cigarette smoke, since it is simpler, less expensive, and better suited for modeling stable COPD [34]. Some studies show that an increased number of cells in the bronchoalveolar lavage, increased expression of IL-6, and emphysematous changes in the lung parenchyma appear already after 1 month of exposure to cigarette smoke at an exposure intensity of 0.00103 cig/L/min and a daily dose of 0.124 cig/L [35], whereas in our case the intensity and dose values were 0.00278 cig/L/min and 0.11 cig/L, respectively.

In our experiment, rats receiving tobacco aerosol demonstrated moderate morphological changes in the lungs. Nevertheless, according to Nitta N.A., et al., with a 6-month exposure of mice to tobacco aerosol, the changes were comparable to those observed in individuals receiving cigarette smoke inhalations. In addition, against the background of exposure to tobacco aerosol, the number of neutrophils and lymphocytes in the bronchoalveolar lavage significantly increased [36]. Thus, it is likely that the induction of a COPD-like phenotype using tobacco aerosol should take more time, and a 6-week exposure was insufficient for the development of pronounced morphological disorders.

Against the background of the action of cigarette smoke and tobacco aerosol, an increase in the expression of most COPD marker genes in lung tissues was recorded. At the same time, unlike other genes, the expression of *CCR3* did not increase. This feature may be due to the fact that in the work of Guan Q., et al. [14], for modeling COPD, exposure to cigarette smoke was combined with nasal instillation of an infectious pathogen (*Klebsiella pneumoniae*), whereas our model did not provide for additional stimulation with live bacteria or components of their cell wall. In addition, we were unable to assess the expression of the *VWF* gene, which may be due to the characteristics of the primers and other reagents used for RT-PCR.

It is important to emphasize that this work was aimed not only at modeling COPD but also at verifying the increased transcription of *TRPM8* in specific models in rats, with the aim of further studying the role of this cation channel in the development of the disease. It is important to note that earlier overexpression of *TRPM8* was recorded either with short-term exposure to cigarette smoke, which is not capable of causing typical COPD-like changes in lung tissue [8], or, conversely, in a model with probably excessive exposure (60 cigarettes per day, 40 days), although detailed experimental conditions (for example,

the volume in which the obtained smoke volume was enclosed) are not specified [37]. We were the first to show that the expression of *TRPM8* mRNA increases not only upon contact with cigarette smoke but also against the background of exposure to tobacco aerosol. Currently, data on the ability of tobacco aerosol components to cause *TRPM8* activation are absent. Nevertheless, it is known that this aerosol has an even more pronounced ability to induce oxidative stress in bronchial epithelial cells in vitro compared with cigarette smoke [38]. Thus, it can be expected that both cigarette smoke and tobacco aerosol will act as activators of this cation channel. The discovered increase in *TRPM8* expression represents an important prerequisite for assessing the potential of these channels as a pharmacological target, which, in turn, creates a basis for further studies aimed at studying their functional role in the pathogenesis of COPD and the development of new approaches to the treatment of this disease.

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Conflicts of interest

The authors declare no conflicts of interest.

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