

Flow Cytometric Study of Mitochondrial Function in ex-Smokers With COPD in Peripheral Blood Mononuclear Cells

EDUARD SERGEEVICH BELSKIKH¹, OLEG MIKHAILOVICH URYASYEV², VALENTINA IVANOVNA ZVYAGINA³, OLGA NIKOLAEVNA ZHURINA⁴, VALERY GRIGOREVICH DEMIKHOV⁵, MIKHAIL IVANOVICH TOKAR⁶.

¹Ryazan State Medical University 9 Vysokovolt'naya st., Ryazan, Russian Federation, 390026

²BioChemMak Diagnostics

¹Assistant, Faculty Therapy with the course of therapy of faculty of additional postgraduate education, Ryazan State Medical University, Russia

²Vice-Rector for Academic Affairs, MD, Head of the Department of Faculty Therapy with a course of Therapy of the faculty of additional postgraduate education, Ryazan State Medical University, Russia

³Associate Professor of the Department of Biological Chemistry with a course of clinical laboratory diagnostics of the faculty of additional postgraduate education Ryazan State Medical University, Russia

⁴Head of the Department of Clinical Laboratory Diagnostics at the Scientific and Clinical Center for Hematology, Oncology and Immunology at the Federal State Medical University of Ryazan State Medical University,

⁵Professor, Director of the Scientific and Clinical Center for Hematology, Oncology and Immunology at the Ryazan State Medical University,

⁶ZAO BioHimMak Diagnostics (BioHimMak Diagnostics, JSC)

Correspondence to Dr. Eduard Sergeevich Belskikh, Russia, Lipetsk, e-mail: ed.bels@yandex.ru, Cell: +7(920)979-68-46

ABSTRACT

Aim: Objective. to assess the level of the mitochondrial membrane potential of peripheral blood mononuclear leukocytes in ex-smokers with non-infectious exacerbations of COPD.

Methods: The 31 subjects were divided into the control group of healthy non-smokers (n = 8), groups of patients with COPD divided into COPD 2 (n = 10) and COPD 3 (n = 13), and into smokers (n = 10) and ex-smokers (n = 13). Blood was taken on the 2-nd day. BD Vacutainer CPT tubes were used to isolate mononuclear cells. Determination of the mitochondrial membrane potential ($\Delta\psi$) of mononuclear leukocytes was performed using cationic dye JC-1 and antibodies to CD45 using flow cytometry.

Results: Patients with COPD compared with the control group are characterized by a decrease $\Delta\psi$ in all studied cell populations (-25% $\Delta\psi$ lymphocytes, p = 0.0023; -23% $\Delta\psi$ leukocytes, p = 0.0063; -28% $\Delta\psi$ monocytes, p = 0.01). The decrease in $\Delta\psi$ of mononuclear leukocytes did not depend on the severity of bronchial obstruction in patients with COPD. The lowest values of $\Delta\psi$ were detected in leukocytes and lymphocytes of smokers with COPD 2 (-35% $\Delta\psi$ lymphocytes, p = 0.032; -34% $\Delta\psi$ leukocytes, p = 0.035) and COPD3 (-35% $\Delta\psi$ lymphocytes p = 0.009; -36% $\Delta\psi$ leukocytes, p = 0.01) compared with control group. $\Delta\psi$ level of ex-smokers was not statistically significantly different from the control.

Conclusion: Smoking cessation is associated with an improvement in the functioning of mitochondria of mononuclear peripheral blood leukocytes in patients with non-infectious exacerbations of COPD.

Keywords: Mitochondria, COPD, flow cytometry, smoking cessation, mononuclear leukocytes.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease with complex pathogenesis, in which, under the action of toxic particles and gases, bronchi of various sizes are involved in pathological changes, which leads to the development of obstructive bronchitis and bronchiolitis with persistent airflow restriction, and pulmonary parenchyma, which leads to destruction of alveoli and the development of emphysema^{1,2}.

In the pathogenesis of COPD, several mechanisms are distinguished. Among them, for example, the enhanced inflammatory response to the action of toxic particles entering with tobacco smoke is associated with an increase in the number of neutrophils, lymphocytes and macrophages in the alveoli and airways of various calibers^{1,2,3}. Along with this, great importance is attached to the imbalance between increased activity of matrix metalloproteinases and a decrease in the level of proteinase inhibitors. This mechanism, both genetically determined (α 1-antitrypsin deficiency) and mediated by the development of an inflammatory response, contributes to the development of emphysema due to the active destruction of the elastic fibers of the walls of the alveoli^{1,2,4}. Separately, the role of oxidative stress, leading

to cell dysfunction or cell death, is highlighted, which is accompanied by damage to the pulmonary extracellular matrix⁴⁻⁷. Oxidative stress develops as a result of the ineffectiveness of the endogenous antioxidant defense system in conditions of excess oxidants: exogenous - inhaled, and endogenous, formed in inflammatory cells and epithelial cells^{4,5,8}. Advances in studying the processes of cell aging and apoptosis suggest a fourth mechanism, a violation of the relationship between the development of apoptosis and the purification of apoptotic cells in the lungs^{3,8,9,10}. Given that the severity of pathological changes in COPD varies greatly from patient to patient, an increased level of apoptosis of respiratory tract cells can explain the progression of COPD in a number of patients even after stopping smoking, one of the most important aspects in the management of patients with COPD^{1,3,11,12}.

Currently, the role of mitochondrial dysfunction in the development of COPD is being actively studied, which is associated both with excessive generation of reactive oxygen species with the development of oxidative stress, and with an increased level of apoptosis^{3-6,13}. Pathological changes in the structure of the mitochondria in the epithelial cells of the bronchi, including depletion of the cristae, increased branching, lengthening and swelling of

the mitochondria in ex-smokers with COPD of extremely severe severity have been revealed³. It has been established that prolonged exposure to cigarette smoke alters the level of markers of mitochondrial biogenesis, oxidative phosphorylation, increases the indicators of oxidative stress of proinflammatory mediators^{2,3}. A study of the smooth muscle cells of the airway wall from patients with COPD demonstrated a decrease in the mitochondrial membrane potential, ATP content, and an increased level of generation of reactive oxygen species^{3,8}. Working with models of oxidative stress caused by chronic ozone exposure in mice revealed the ability of mitochondrial antioxidant MitoQ to restore mitochondrial function in lung tissue, which was associated with preventing the development of airway hypersensitivity and reducing the number of inflammatory cells and mediators [8]. These results demonstrate that mitochondrial dysfunction may play a key role in the pathogenesis of COPD and potentially provide a new therapeutic goal for maintaining and preventing COPD^{5,8,10}.

Patients with COPD are characterized not only by lung damage, but also by systemic manifestations. Mitochondrial dysfunction under prolonged exposure to cigarette smoke has also been found to develop outside the lungs - in skeletal muscle, vascular wall, blood cells^{7,13,14}. The isolation and study of the mitochondrial function of blood cells, such as lymphocytes and monocytes, directly from patients is more accessible than a biopsy for obtaining smooth muscle cells of the bronchial wall and allows to get these cells in sufficient quantity to study, which does not require their additional cultivation.

The functioning of the respiratory chain complexes is associated with the formation of transmembrane difference in electrochemical potentials on the inner mitochondrial membrane. A decrease in mitochondrial membrane potential indicates impaired mitochondrial function and the possible development of apoptosis and can be assessed by flow cytometry using cationic fluorescent dyes, which allow to detect depolarization of mitochondrial membranes [8,15,16]. One of the most common methods for determining changes in transmembrane potential and imaging mitochondria with low and high membrane potentials is flow cytometry using cationic dye JC-1 (5,5', 6,6'-tetrachloro-1,1', 3,3' -tetraethylbenzimidazole carbocyanine iodide / chloride)⁸.

Mitochondrial dysfunction of patients with COPD, closely associated with oxidative stress and proapoptotic processes, may become a new goal for the treatment and prevention of COPD progression [5,8]. At the same time, the study of indicators of mitochondrial functions in patients with COPD with varying degrees of severity, severity of symptoms, smoking history, can contribute to the selection of groups of patients for the selection of the optimal treatment regimen.

Thus, the goal of the work is to study the membrane potential of mitochondrial lymphocytes and peripheral blood monocytes using cationic dye JC-1 using flow cytometry in patients with chronic obstructive pulmonary disease.

The following tasks were set: to study the level of mitochondrial membrane potential as a marker of mitochondrial dysfunction and determine the effect of

smoking cessation on the level of mitochondrial membrane potential of blood lymphocytes and monocytes in patients with exacerbation of chronic obstructive pulmonary disease in comparison with healthy volunteers.

METHODS

The pilot study was performed in the period from 2017 to 2018 at the Department of Intermediate Level Therapy of acad. I.P. Pavlov State Medical University of Ryazan. The study was approved by Local Ethics Committee of Ryaz SMU (protocol № 2 от 7.10.2016) and complies with the requirements of Good Clinical Practice (GCP) and the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects".

The basis for inclusion in the study was signed informed consent. The inclusion criteria for patients with chronic obstructive pulmonary disease (COPD) were age from 40 to 75 years, the initial post-bronchodilation FEV₁/FVC index was < 0.7. Exclusion criteria for all groups were surgical interventions on the lungs in history, alcohol and drug abuse, patients with lung diseases other than COPD, or those with significant inflammatory diseases, other chronic diseases of internal organs in the decompensation phase. Investigated aged 40 to 80 years (62 ± 11 years) were divided into several groups. Clinical and demographic characteristics of the study are shown in Table 1.

The minimal sample size was calculated using Open Epi calculator with the statistical assumptions of 5% alpha error and 95 % confidence interval taking frequency of decreased mitochondrial membrane potential of leukocytes at least on 28% to be 98.74% amongst patients with COPD [17]. The first group was the control group, which included 8 non-smoking volunteers without concomitant chronic respiratory diseases. In accordance with the GOLD spirometry classification, patients with chronic obstructive pulmonary disease (COPD) hospitalized due to non-infectious exacerbations were divided into a group of patients with COPD 2 (50% < forced expiratory volume in 1 second (FEV₁) < 80%) (n = 10) (group 2) and a group of COPD 3 (30% < FEV₁ < 50%) (n = 13) (group 3). In relation to smoking, the studied patients with COPD were divided into a group of smokers (group 4, n = 10) and ex-smokers (group 5, n = 13), who quit smoking more than a year ago. Spirometry was performed using a Micro Lab mk 8 spirometer (Micro Medical, UK), pulse oximetry using a SpiroTel SpO₂ (MIR, Italy). Blood sampling was carried out in the morning on an empty stomach on the second day of hospitalization by venipuncture using test tubes containing sodium heparin, separating gel and ficoll solution to create a density gradient (BD Vacutainer CPT, USA).

Isolation of mononuclear leukocytes was performed by centrifuging for 16 minutes at 1600 G. The resulting cell suspensions were washed three times with warm (37°) phosphate-saline buffer (pH 7.4). The cells resuspended in the buffer were counted on a Sysmex XT-2000i hemocytometer (Japan) and diluted to a concentration of 10⁶ / ml. To study the mitochondrial membrane potential ($\Delta\psi$), the MitoProbe™ JC-1 Assay Kit (Molecular Probes, USA) was used. Prior to incubation, antibodies to CD45

conjugated with fluorochrome (CD45-PC-5, Beckman Coulter, USA) were added to the stained cells before incubation to further identify populations of mononuclear leukocytes. incubation was carried out in a thermostat for 20 minutes at 37°. Stained cells were washed once, and then subjected to flow cytometry on a Cytomics FC 500 (USA) cytometer using CXP software. The method used is based on the ability of the monomer 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocytin iodide (JC-1) to accumulate depending on the level of Δψ inside the mitochondria with the formation of aggregates characterized by fluorescent emission with the intensity maximum in the red-orange spectrum (λ = 590 nm), which was measured on a FL-2 channel of the flow cytometer. The monomers JC-1 are characterized by a maximum fluorescence intensity in the green spectrum (λ = 529 nm), which was measured on the FL-1 channel. In the studied samples, the ratio of the intensity of red fluorescence to green (FL-2 / FL1; Red / Green) was determined, which was taken as the relative value of the potential difference of the inner mitochondrial membrane (Δψ, mitochondrial membrane potential). The ratio of the intensity of red fluorescence to green was determined in regions identified by the indicators of lateral light scattering (SS) and signal intensity of the fluorescent label to CD45 on the FL-4 channel (Figure 1). The magnitude of the FL2/FL1 events in region A was considered as the mitochondrial membrane potential of lymphocytes, in region D-Δψ of all mononuclear leukocytes, in the region C-Δψ of monocytes. A control sample with the mitochondrial respiration CCCP disrupter was placed for each study (Fig. 2).

Statistical processing of the results was carried out using the StatPlus 6.0. The correspondence of the samples to the normal distribution was checked by means of the Shapiro – Wilk criterion. To determine statistically significant differences of independent samples on a quantitative basis in the case when the distribution was different from the normal or the result of the Leuven test indicated that there is a difference in dispersions, the Mann-Whitney test for comparing the two groups, and for groups with a normal distribution and no difference in dispersions - univariate analysis of variance and the Newman-Keuls test for multiple comparisons and Student's test for comparing the two groups. For multiple comparison

of groups with abnormal distribution, the Kruskal-Wallis test was used. Analysis of the correlation relationships in samples with abnormal distribution was carried out using Spearman's coefficient of rank correlation, in samples with normal distribution -with Pearson's coefficient. Differences were considered statistically significant when the probability of the null hypothesis of the absence of differences was p<0.05.

RESULTS

Patients with the control group compared with the control group represent a decrease in the level of mitochondria in all studied cell populations (Fig. 3) (-25% Δψ gate A, p = 0.0023; -23% Δψ gate D, p = 0.0063; - 28% Δψ gate C, p = 0.01).

Groups of patients with COPD with varying severity of bronchial obstruction did not statistically significantly differ from each other (Table 2). At the same time, Δψ of mononuclear cells of groups such as COPD 2 (-17% Δψ gate A, p = 0.04; -32% Δψ gate C, p = 0.022), and COPD 3 (-32% Δψ gate A, p = 0, 0012; -28% Δψ gate D, p = 0, 0071; -24% Δψ gate C, p = 0.04) were reduced compared to the control.

Evaluation of the level of Δψ mitochondria of mononuclear cells in subgroups separated depending on the severity of obstruction and smoking status revealed that smokers with COPD 3 GOLD (-35% Δψ gate A, p = 0.009; -36% Δψ gate D, p = 0.01) and COPD 2 GOLD (-35% Δψ gate A, p = 0.032; -34% Δψ gate D, p = 0.035) have decreased rates compared with the control group. At the same time, the Δψ level of mitochondria of ex-smokers' mononuclear cells in groups with different severity of broncho-obstruction was not statistically significantly different from the control (Table 3).

Correlation analysis revealed a connection of moderate strength between Δψ of different populations of peripheral blood mononuclear cells and clinical and functional indicators of patients with COPD: with FEV₁ (gate A, r = 0.52, p=0.002; gate D, r = 0.46, p=0.009), SpO₂ (gate A, R = 0.61, p=0.0003; gate D, R = 0.58, p=0.0006), pack-years index (gate A, R = -0.44, p=0.013; gate D, R = -0.48, p=0.007).

Table 1:

Indicator	Control, n=8 (group 1)	COPD 2, n=10 (group 2)	COPD 3, n=13 (group 3)	p
Age, M±s, year	53.87±13.53	65.4±11.15	65.15±6.68	
male	5	7	12	
female	3	3	1	
FEV ₁ , M±s, %	91.5±1.41	70.3±10.35	44.23±6.43	p _{1,2} =0.000125 p _{1,3} =0.000125 p _{2,3} =0.000127
smokers	0	4	6	
ex-smokers	0	6	7	
never smokers	8	0	0	
Index pack-year, Me[Q1;Q3] %	0	30[25;30]	25[20;25]	
SpO ₂ , Me[Q1;Q3] %	97[97.98]	96[94.3;96]	92[92;96]	p _{1,3} =0.0013

Table 2: Changes in mitochondrial membrane potential depending on smoking.

Groups	Red/Green, Ly45(gate A)	Red/Green, Leu45(gate D)	Red/Green, Mon45 (gate C)
Control, n=8 [1]	0.262±0,026 p ₁₋₂ =0.0002	0.242±0.032 p ₁₋₂ =0.0003	0.173±0,052 p ₁₋₂ =0.047 p ₁₋₃ =0.025
COPD smoker, n=10 [2]	0.163±0.026 p ₂₋₃ =0.0046	0,151±0,026 p ₂₋₃ =0,0089	0.124±0.036
COPD ex-smoker, n=13 [3]	0.221±0.056	0.210±0.053	0.127±0.044

Note: Red / Green - the ratio of red-orange to green fluorescence, which determines the level Δψ of mitochondria of the studied cells; region A includes lymphocytes isolated according to gate Ly CD 45, region C includes monocytes isolated from gate Mon CD 45, region D includes mononuclear leukocytes isolated from gate Lymph CD 45 and gate Mon CD 45.

Table 3: Differences in the mitochondrial membrane potential of smokers and ex-smokers with varying severity of bronchial obstruction.

Groups	Red/Green, Ly45 (gate A)	Red/Green, Leu45 (gate D)	Red/Green, Mon45 (gate C)
COPD2 smoker, n=4 [1]	0.168[0.159;175] p ₁₋₅ =0.032	0.161[0.149;169] p ₁₋₅ =0.035	0.101[0.087;0.122]
COPD2 ex-smoker, n=6 [2]	0.267[0.221;0.269]	0.226[0.200;0.254]	0.120[0.105;0.146]
COPD3 smoker, n=6 [3]	0.168[0.132;0.179] p ₁₋₅ =0.009	0.158[0.119;0.183] p ₁₋₅ =0.01	0.146[0.129;0.159]
COPD3 ex-smoker, n=7 [4]	0.209[0.146;0.234]	0.204[0.145;0.256]	0.144[0.083;0.167]
Control, n=8 [5]	0.260[0.247;0.276]	0.245[0.247;0.276]	0.164[0.129;0.214]

Note: Red / Green - the ratio of red-orange to green fluorescence, which determines the level Δψ of mitochondria of the studied cells; region A includes lymphocytes isolated according to gate Ly CD 45, region C includes monocytes isolated from gate Mon CD 45, region D includes mononuclear leukocytes isolated from gate Lymph CD 45 and gate Mon CD 45.

Fig. 1: Isolation of mononuclear leukocytes and their subpopulations with determination of the red /green fluorescence ratio

Fig. 1 Note: SS is an indicator of lateral light scattering, CD45-PC5 is fluorescence of labeled antibodies to CD45, region A includes lymphocytes isolated according to gate Ly45, region C includes monocytes isolated according to gate Mon45.

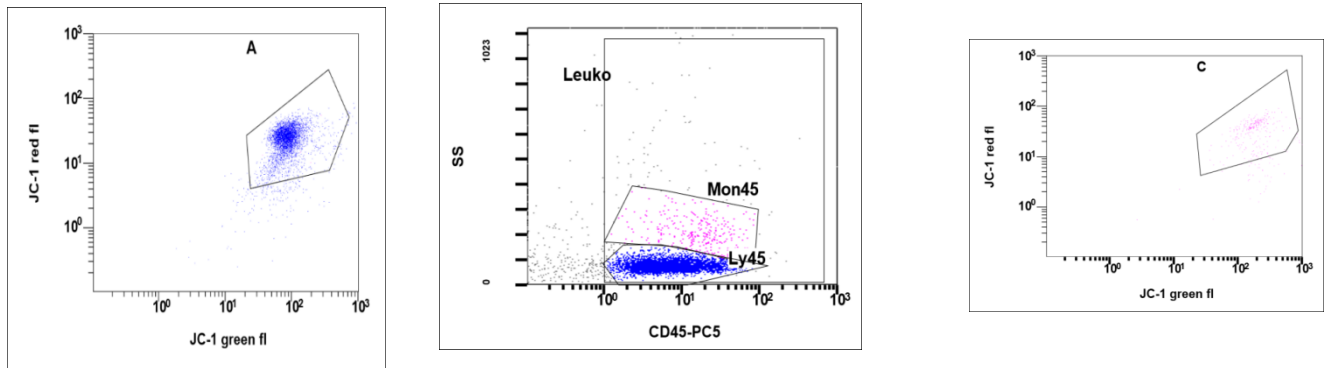


Fig. 2: A sample with (left) and without (right) the CCCP (carbonyl cyanide-2-chlorophenylhydrazone) - a disruptor of mitochondrial membrane metabolism, served for each test sample of JC-1 as control

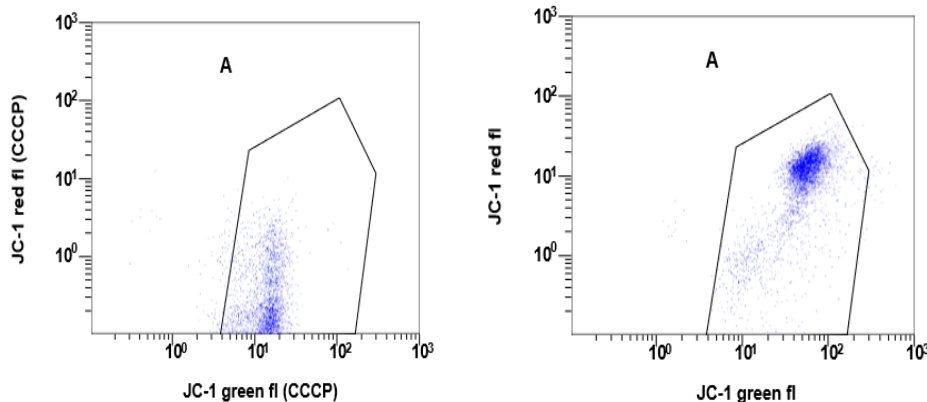


Fig. 3 Note. Difference in mitochondrial membrane potential of mononuclear cells in COPD and Control groups. Note: FL2 (Red) / FL1 (Green) - the ratio of red-orange to green fluorescence, which determines the level Δψ of mitochondria of the studied cells; region A includes lymphocytes isolated according to gate Ly CD 45, region C includes monocytes isolated from gate Mon CD 45, region D includes mononuclear leukocytes isolated from gate Lymph CD 45 and gate Mon CD 45; *Gate A, p_{COPD-Control} = 0,0023; **Gate D, p_{COPD-Control} = 0,0063; ***Gate C p_{COPD-Control} = 0,01.

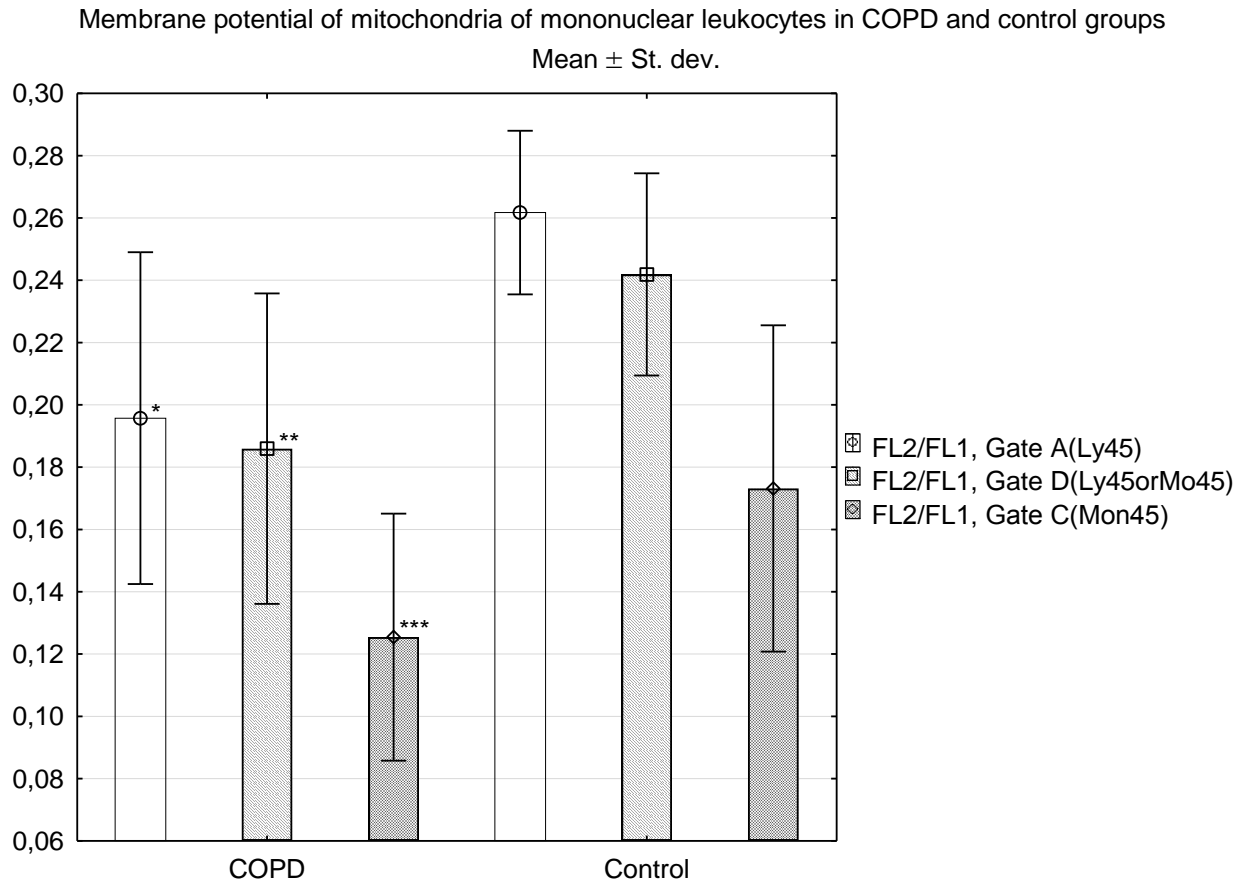
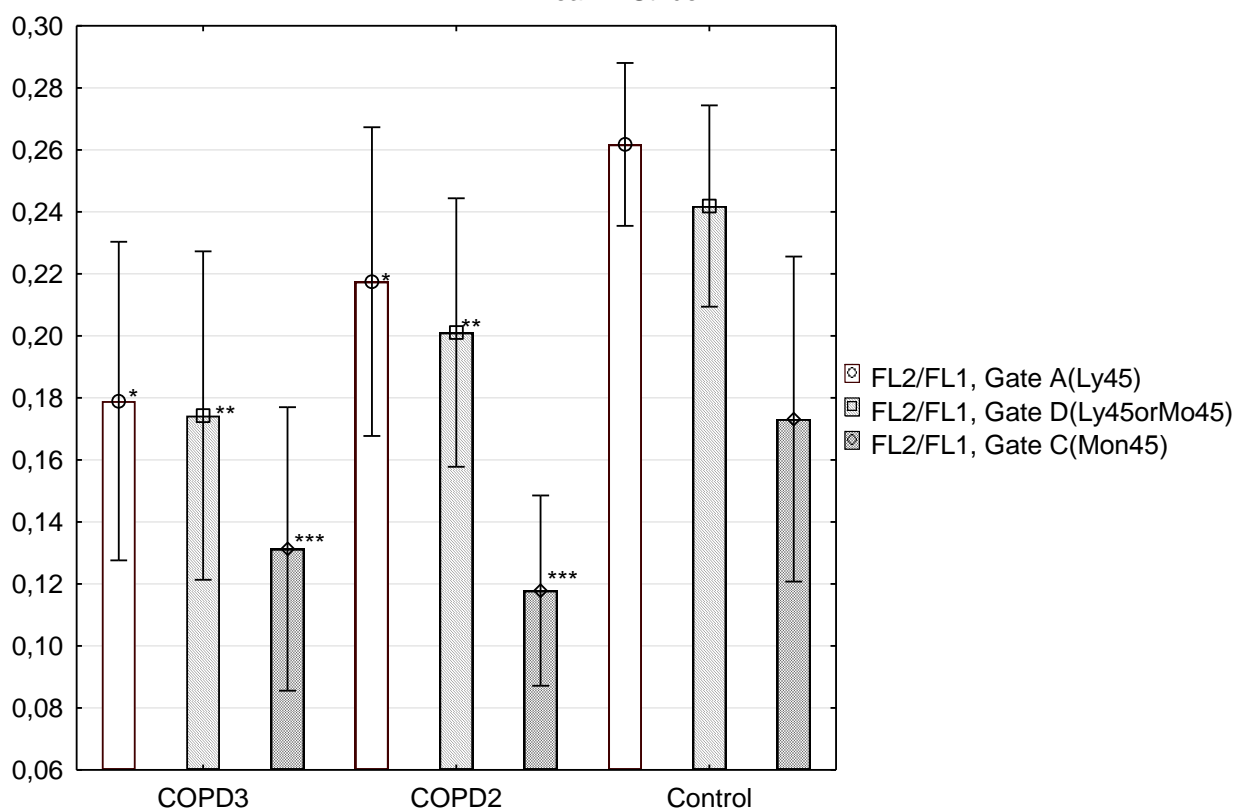


Fig. 4 Note. Difference in mitochondrial membrane potential of mononuclear cells in COPD and Control groups in depending on FEV₁ (table 3) Note: FL2 (Red) / FL1 (Green) - the ratio of red-orange to green fluorescence, which determines the level $\Delta\psi$ of mitochondria of the studied cells; region A includes lymphocytes isolated according to gate Ly CD 45, region C includes monocytes isolated from gate Mon CD 45, region D includes mononuclear leukocytes isolated from gate Lymph CD 45 and gate Mon CD 45; *Gate A $p_{\text{Control-GOLD2}}= 0,04$; $p_{\text{Control-GOLD3}}= 0,0012$; **Gate D $p_{\text{Control-GOLD3}}= 0,0071$; Gate C $p_{\text{Control-GOLD2}}=0,022$, $p_{\text{Control-GOLD3}}=0,04$.

Mitochondrial membrane potential in mononuclear leukocytes in depending on FEV₁Mean \pm St. dev.

DISCUSSION

The revealed decrease in the mitochondrial membrane potential of mononuclear leukocytes is a prerequisite for the disruption of the functioning of this type of cells and the development of proapoptotic processes in them during non-infectious exacerbation of COPD. The results suggest that it is likely that the change in mitochondrial potential is not directly related to the degree of bronchial obstruction. At the same time, a significant contribution to the development of mitochondrial lymphocyte dysfunction in COPD contributes to the chronic effects of cigarette smoke. The revealed lack of statistically significant differences between $\Delta\psi$ lymphocytes of healthy control volunteers and ex-smokers of patients with COPD2 GOLD may indicate reversibility of mitochondrial dysfunction changes at some stages of the disease, which requires further study on a larger sample.

The moderate closeness of the SpO₂ and $\Delta\psi$ connection of the studied cell populations may be due to different adaptability in the development of respiratory failure. Almost close relationship between $\Delta\psi$ lymphocytes and the status of ex-smoker (gate A, R = 0.70; gate D, R = 0.697), as well as the absence of statistically significant differences between the studied indicators of the control group and patients – ex-smokers suggests that it is likely that smoking cessation can reduce the severity of mitochondrial dysfunction of peripheral blood mononuclear

leukocytes in patients with COPD with non-infectious exacerbations.

Conclusions.

Flow cytometry using JC-1 cationic dye can be used to detect mitochondrial dysfunction of mononuclear leukocytes in patients with chronic obstructive pulmonary disease. Smoking cessation is associated with an improvement in the functioning of mitochondria of mononuclear peripheral blood leukocytes in patients with non-infectious exacerbations of COPD.

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