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Morphofunctional Characteristics of Erythrocytes and Blood Erythropoietin Level in Patients as Predictors of Severe Course of COVID-19

V. V. Revin[1], L. A. Balykova[1], O. A. Radaeva[1], V. V. Shchapov[1,2], E. S. Revina[1], S. I. Pinyaev[1], Yu. A. Kostina[1], and E. D. Kozlov[1]

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Morphological and functional characteristics of erythrocytes, hemoglobin, and erythropoietin level in the venous blood were evaluated by laser interference microscopy, Raman spectroscopy with a short-focus extreme aperture lens monochromator, and by ELISA, respectively, in 30 patients with verified moderate COVID-19 at the time of hospitalization and 30 healthy volunteers. The patients whose course of COVID-19 has worsened to critical by day 5 had already had lower (p<0.001) indicators at the time of hospitalization such as the area and thickness of erythrocytes, the hemoglobin distribution and packing density, hemoglobin conformation index $(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$ reflecting its oxygen affinity, and blood erythropoietin content. Our findings suggest that these characteristics of erythrocytes, hemoglobin, and erythropoietin can serve as potential predictors of COVID-19 aggravation in hospitalized patients.

Key Words: *COVID-19; worsening course markers; erythropoietin; erythrocytes; hemoglobin*

Considerable growth of the number of cases of SARS-CoV-2 infection and the number of victims was reported in the Russian Federation in 2021. The risk factors for unfavorable COVID-19 outcomes are the age over 65 years, blood coagulation system dysfunctions, and concomitant cardiovascular pathology [14]. Some researchers argue that the violation of the morphofunctional properties of erythrocytes and disturbances of iron metabolism also determine the severity of COVID-19 course [4,13]. Non-structural proteins of SARS-CoV-2 (orf1ab, ORF10, ORF3a, and ORF8) disrupt the transport function of hemoglobin (Hb), displacing ferrous iron atoms from the porphyrin core of the β-chain [8]. High levels of proinflammatory cytokines inhibit erythroid progenitor cells, altering their morphology and shortening their lifespan [7]. On the other hand, erythropoietin (EPO) can have a protective effect, exhibiting antiapoptotic and cytoprotective properties in pathological conditions associated with COVID-19 [12]. However, the role of indicators of the structure and function of erythrocytes and Hb as risk factors for severe course and/or progression of coronavirus infection has not been determined.

We analyze the morphological and functional characteristics of erythrocytes, Hb, as well as the EPO content in the blood of patients with COVID-19 to identify their pathogenetic interactions with the disease activity indicators.

[1]N. P. Ogarev National Research Mordovia State University, Saransk, Mordovia Republic, Russia; [2]S. V. Katkov Republican Clinical Hospital, Saransk, Mordovia Republic, Russia. ***Address for correspondence:*** dr.kozlov@yandex.ru. E. D. Kozlov

MATERIALS AND METHODS

We performed a comprehensive trial consisting of prospective and retrospective stages and including 30 patients (15 men and 15 women, mean age 68 years (95%CI 57-82%), hospitalized with verified COVID-19 between March 1 and May 25, 2021. The inclusion criteria were age 18-80 years, moderate severity of COVID-19 at the time of admission. The patients treated for COVID-19 before hospitalization, patients with mild/severe COVID-19 at the time of hospitalization, as well as patients with diabetes mellitus (types 1 or 2) with multiple complications, uncontrolled bronchial asthma, decompensated liver failure, terminal renal failure, functional class III-IV chronic heart failure were excluded from the study. The control group was presented by age-matched healthy participants without signs of acute pathologies or exacerbation of chronic diseases, or COVID-19 at present and in the past (PCR-negative, IgM- and IgG-negative), examined within the framework of regular health check-up at the outpatient department. The study protocol was reviewed and approved by the Ethical Committee of the N. P. Ogarev National Research Mordovia State University (Protocol No. 12, December 14, 2008, and Protocol No. 85, May 27, 2020). All patients signed informed consent for participation in the study.

We analyzed correlations of the degree of oxygen saturation of the blood (pO_2), CT changes (percentage of lung damage), serum levels of C-reactive protein (CRP), fibrinogen, D-dimer, and activated partial thromboplastin time (APTT) with morphofunctional characteristics of erythrocytes, Hb, and EPO content in the blood.

In the venous blood taken on the first day of hospitalization before the start of combined therapy, and the morphological and functional characteristics of erythrocytes, Hb, and EPO content were evaluated. Morphological changes in erythrocytes and redistribution of Hb were examined by laser interference microscopy using a MII-4M laser interference microscope (LOMO). The oxygen-binding properties of Hb were analyzed by Raman spectroscopy on an InViaBasis spectrometer with a short-focus extreme aperture lens monochromator (Renishaw). The changes in the oxygen-binding properties of erythrocytes were tracked by the characteristic Raman spectra bands (maximum positions indicated): 1355, 1375, 1550, 1580, 2850, 2880 and 2930 cm^{-1}. The following parameters were determined: the ratio of the Raman intensity bands 1375 to 1355 cm^{-1} (I_{1375}/I_{1355}) characterizing the relative number of Hb complexes with ligands, mainly the oxyhemoglobin content; I_{1355}/I_{1550} characterizing the relative ability of Hb in the sample to bind ligands (including oxygen); $(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$ showing the affinity of Hb to ligands, primarily to oxygen; $I_{1375}/(I_{1355}+I_{1375})$ reflecting the relative amount of oxyhemoglobin in the blood; I_{1580}/I_{1550} characterizing the contribution of vibrations of methine bridges in hematoporphyrin (which characterizes the affinity of Hb to ligands, particularly to oxygen); I_{2850}/I_{2880} characterizing the contribution of symmetric fluctuations to asymmetric fluctuations of amino acid methylene groups; I_{2930}/I_{2850} reflecting the contribution of symmetric fluctuations of terminal methylene groups to symmetric fluctuations of amino acid methylene groups (characterizes the change in the polarity of the amino acids surrounding). The EPO level in the blood serum was assessed using ELISA test systems (measurement range 1.2-156 mU/ml).

Statistical data processing was carried out using Statistica 10.0 software (StatSoft, Inc.). The quantitative data were presented as median (Me) and 95% confidence interval (95%CI). The normality of the distribution was tested using the Kolmogorov–Smirnov test. The results substantiates the use of nonparametric Wilcoxon's and Mann–Whitney tests to compare the parameters; the correlations between the variables were assessed using the Spearman's correlation coefficient (r).

RESULTS

In COVID-19 patients, I_{1375}/I_{1355} ratio characterizing the relative number of oxyhemoglobin complexes and $(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$ ratio reflecting Hb affinity to oxygen were reduced in comparison with control group (Table 1). We also observed a decrease in the EPO content and morphofunctional characteristics of erythrocytes (area, phase volume, optical path difference, and Hb packing density) in COVID-19 patients in comparison with healthy individuals (Table 1).

One-way correlation analysis revealed no significant relationships between the morphological and functional characteristics of erythrocytes and clinical and laboratory data of the patient at the time of hospitalization. The mean time from admission to the hospital to COVID-19 worsening requiring intensive care was 5.8 (95%CI (53-10.7)) days. This fact suggested to repeat the correlation analysis of the morphological and functional characteristics of patient erythrocytes and Hb obtained at the time of hospitalization with clinical and laboratory characteristics of the patients on day 5 of inpatient treatment to determine the predictive potential of these characteristics in relation to the progression of COVID-19. The repeated analysis revealed significant negative correlations between erythrocyte thickness and Hb packing density at the time of the patient hospitalization with the degree (%) of the pulmonary parenchyma involvement according

TABLE 1. Morphofunctional Characteristics of Erythrocytes, Hemoglobin, and EPO Content in COVID-19 Patients and Healthy Subjects (Me (95%CI))

Parameter	Control group	Patients with COVID-19
I_{1375}/I_{1355}	0.94 (0.92-1.07)	0.91 (0.86-0.93)*
$(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$	1.22 (1.18-1.24)	0.90 (0.85-0.92)***
EPO, mU/ml	112 (108-117)	94.7 (91.3-99.2)**
Erythrocyte area, μm^2	62.2 (58.4-67.6)	49.6 (47.5-53.9)**
V_{phase}, μm^3	70.5 (61.7-88.3)	37.3 (29.6-42.1)**
Optical-path difference of erythrocytes, nm	79.6 (71.4-87.6)	57.6 (48.3-61.7)**
Hb packing density, pg	24.1 (22.6-27.5)	12.9 (10.4-13.7)**

Note. *p<0.05, **p<0.01, ***p<0.001 in comparison with the control group.

to CT data (r=0.79 and r=0.81, respectively p<0.001) and with pO_2 on day 5 of inpatient treatment (r=0.81 and r=0.82, respectively, p<0.001). In addition, erythrocyte thickness inversely correlated with D-dimer level (r=-0.85, p<0.001). A positive correlation between the blood EPO content and erythrocytes thickness (r=0.778, p<0.001) at the time of hospitalization and a negative correlation between the blood EPO content and pO_2 on day 5 of inpatient treatment (r=-0.75, p<0.001).

These data actualized retrospective analysis of the morphofunctional characteristics of erythrocytes and Hb with consideration for the severity of COVID-19 on days 5-6 of inpatient treatment (Table 2). The patients with moderate course of COVID-19 at the time of admission and progressing to severe or extremely severe on day 5 of inpatient treatment, had lower area and thickness of erythrocytes (p<0.001), lower phase volume of erythrocyte (V_{phase}) (p<0.001), lower packing density of Hb (p<0.001), as well as lower Hb conformation index $(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$ reflecting its affinity to oxygen and lower blood EPO content (p<0.001) at the time of admission. These findings allow considering these parameters as prognostic markers of unfavorable course (progression) of COVID-19 in hospitalized patients. No correlations between the studied parameters and the levels of CRP, fibrinogen, and APTP were found (p>0.05).

The reports published during the pandemics have demonstrated the role of erythrocyto- and hemoglobinopathies aggravating hypoxemia and hypoxia as well as cell overload with iron in the pathogenesis of COVID-19 [9].

The observed structural abnormalities of erythrocytes are probably associated with changes in activity of membrane-bound enzymes, membrane glycoproteins, and redistribution of the charge on the erythrocyte surface, which can promote erythrocyte aggregation, deterioration of the rheological properties of blood, and involvement of the vascular endothelium into the pathological process, which is an important component of pulmonary pneumonitis [1] and cardiovascular complications [2].

Some authors [3] argue that damage to erythrocytes in COVID-19 occurs due to immune-mediated

TABLE 2. Morphological and Functional Predictor Characteristics of Erythrocytes, Hb, and EPO Content in Blood of Patients with COVID-19 of Varying Severity during the Follow-Up Period, Me (95%CI)

Parameter	Patients with COVID-19 (n=30)			Control group (n=10)
	moderate severe (n=16)	severe (n=10)	extremely severe (n=4)	
I_{1375}/I_{1355}	0.94 (0.93-1.06)	0.93 (0.92-0.96)	0.94 (0.92-0.98)	0.94 (0.91-1.07)
$(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$	0.91 (0.78-0.93)	0.93 (0.9-0.94)	0.88 (0.87-0.90)**$^{++}$	1.22 (1.18-1.24)**$^{++oo}$
Erythrocyte area, μm^2	55.1 (44.6-59.3)	49.3 (44.6-51.7)*	44.1 (40.2-46.7)**$^{++}$	62.2 (58.4-67.6)**$^{++oo}$
Erythrocyte thickness, μm	0.93 (0.84-0.99)	0.83 (0.78-0.92)	0.67 (0.5-0.86)**$^{++}$	1.14 (1.02-1.25)**$^{++oo}$
V_{phase}, μm	39.1 (36.7-40.9)	37 (36.6-39.7)	31.2 (27.7-38.3)**$^{++}$	70.5 (61.7-88.3)**$^{++oo}$
Hb packing density, pg	14.7 (13.2-14.7)	12.3 (12.1-13.2)	11.9 (13.3-12.3)**	24.1 (22.6-27.5)**$^{++oo}$
EPO, mU/ml	112 (88.7-117)	86.3 (74.5-91.7)*	57.8 (32.2-63.1)**$^{++}$	112 (108-117)**$^{++oo}$

Note. *p<0.01, **p<0.001 in comparison with the moderate course; ^{++}p<0.001 in comparison with severe course; ^{oo}p<0.001 in comparison with extremely severe course of COVID-19 (Mann—Whitney test).

mechanisms and/or damage to cells induced by microangiopathy. It is possible that viremia, hypoxia, and immune disorders (antibodies binding to erythrocytes) affect the morphology, rheology, and survival of erythrocytes and contribute to the complex pathogenesis of COVID-19.

Several groups of researchers proposed to use the increase in the red cell distribution width (RDW) in clinical practice during risk stratification of COVID-19 progression. Increased RDW at the time of the diagnosis significantly correlated with putting on mechanical ventilation (p=0.0109) and increased mortality (p<0.0001) [11].

Our results confirm the point that changes in the morphological and functional parameters of erythrocytes is an indicator of severe or extremely severe course and unfavorable prognosis of COVID-19. We believe that such indicators as reduced area and thickness of erythrocytes against the background of reduced Hb packing density and reduced affinity of Hb for ligands (primarily to oxygen) can potentially be used as markers of COVID-19 progression and the need for mechanical ventilation.

Among the factors regulating erythropoiesis and some of immune mechanisms, EPO plays a special place. Normally, tissue hypoxia stimulates the production of EPO [15]. The patients with COVID-19 had reduced EPO content, which was associated with aggravation of the disease course in this study. In addition, EPO antagonizes with proinflammatory cytokines TNFα and IL-1β within the framework of physiological regulation [5], therefore, the revealed decrease in EPO potentially blocks the protective anti-inflammatory mechanisms in patients with COVID-19 [10] and neutralizes its effect on pulmonary edema and pulmonary angiogenesis regulation [6].

The changes in the hematoporphyrin conformation and oxygen-binding properties of Hb disrupt the functional activity of erythrocytes and contribute to aggravation of hypoxia in patients with COVID-19, which was previously seen in patients with cardiovascular diseases [1,2]. The observed decrease in oxyhemoglobin content also correlates with indicators of the aggravation of the course of COVID-19, in particular with saturation on day 5 of hospitalization. That is why, assessment of Hb conformation by Raman spectroscopy can be used as a predictor of the progression of pulmonary injury.

Changes in the morphological and functional characteristics of erythrocytes and Hb, as well as EPO content in the blood, are important pathogenetic components of COVID-19 aggravation. It is important to note that the data obtained are relevant, but more observations are required to determine the specificity and sensitivity of the proposed diagnostic approach (determination of the structure and function of erythrocytes and Hb) for stratification of patients at high risk of COVID-19 progression.

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