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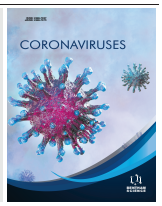
RESEARCH ARTICLE

Ultrastructural Blood Cell Changes in Patients with COVID-19



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Abstract: ***Introduction*:** Nowadays, the coronavirus disease COVID-19 is a global problem for the population of the whole world which has acquired the character of a pandemic. Under physiological conditions, in a healthy person, erythrocytes make up 96% of all blood cells, leukocytes 3%, and thrombocytes about 1%. In healthy individuals, erythrocytes are mostly shaped like a biconcave disc and do not contain a nucleus. The diameter of the erythrocyte is 8 microns, but the peculiarities of the cell structure and the membrane structure ensure their great ability to deform and pass through capillaries with a narrow lumen of 2-3 microns. Therefore, the study of the morpho-functional state of blood cells, namely erythrocytes, in this category of patients is relevant and deserves further research.

***The Aim*:** To figure out the effect of the coronavirus disease COVID-19 on the ultrastructural blood cell changes, in particular erythrocytes, in patients with ischemic heart disease (IHD) and diabetes mellitus type 2.

***Materials and Methods*:** Twelve patients with COVID-19 who had an acute myocardial infarction were examined. The comparison group consisted of 10 people with acute myocardial infarction without symptoms of COVID-19. The average age of the patients was 62 ± 5,6 years. The functional state and ultrastructure of blood cells were studied using electron microscopy.

***Results*:** In the presence of COVID-19, we detected both calcification and destruction of erythrocytes and platelets. Reticulocytes were detected much more often in these individuals than in the comparison group. In patients with acute myocardial infarction in the presence of type 2 diabetes and COVID-19, a significant number of markedly deformed, hemolyzed erythrocytes or with signs of acanthosis, which stuck together and with other destructively changed blood cells, were found. We also detected «neutrophils extracellular traps» (NETs).

***Conclusions*:** Morphological changes of blood cells in COVID-19 varied according to the disease course and severity especially in the background of a weakened immune system in older and elderly people, in the presence of diabetes, excessive body weight, cardiovascular diseases and occupational hazards. Under the influence of COVID-19, blood cells are destroyed by apoptosis and necrosis. Therefore, hypoxia and ischemia of vital organs of the human body occur.

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1. INTRODUCTION

Nowadays, the coronavirus disease COVID-19 is a global problem for the population of the whole world which has acquired the character of a pandemic [1, 2]. In the majority of patients with an average degree of severity of COVID-19, as well as in persons with a severe form of the disease, one of the main syndromes is increasing shortness of breath against the background of a reduced level of hemoglobin and a high content of ferritin in the blood with the appearance of a «cytokine storm», which indicates the formation of the severe hypoxemic syndrome [3-11]. In the process of destruction of hemoglobin by the COVID-19 virus and its non-structural proteins through hemolysis of erythrocytes, leakage of heme from damaged erythrocytes [12] and other hemoproteins containing porphyrin, the level of iron increases in blood serum and causes toxic effects with the formation of oxidative stress [13]. Glycosylated hemoglobin porphyrin is most often subjected to viral capture, the level of which is higher in patients with diabetes mellitus, obesity, cardiovascular diseases, and metabolic disorders, in particular, in older age groups [14-27].

With COVID-19, there is an urgent need for macrophage-mediated exchange of erythrocytes and hemoglobin due to a violation of gas exchange, which leads to the development of tissue hypoxia and systemic hypoxemia. However, an excessive number of damaged erythrocytes and other

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blood cells, heme molecules, elevated levels of iron, ferritin, and many pro-inflammatory proteins (cytokines, C-protein, *etc*.) cause microcirculation disorders, obliteration of blood vessels, and the occurrence of blood clots, which lead to the formation of necrosis [2, 28].

The aim is to figure out the effect of the coronavirus disease COVID-19 on the ultrastructural blood cell changes, in particular erythrocytes, in patients with IHD and diabetes mellitus type 2.

2. MATERIALS AND METHODS

Twelve patients with COVID-19 who had an acute myocardial infarction were examined. These patients were receiving inpatient treatment in the Department of Cardiology and Reperfusion Therapy of the Communal non-commercial enterprise "Clinical Emergency Medical Care Hospital of Lviv". Among them were 8 men and 4 women. The average age of the patients was 62 ± 5.6 years. The comparison group consisted of 10 people with acute myocardial infarction without symptoms of COVID-19. Both groups were compared by age and gender. The examination program included a detailed history collection regarding risk factors for IHD, namely, hypertension, smoking, type 2 diabetes, overweight, dyslipidemia, excessive alcohol consumption, and occupational hazards.

All patients underwent standard laboratory examinations (general analysis of blood and urine and biochemical studies), qualitative determination of markers of myocardial necrosis (myoglobin, CK-MB, troponin I), blood lipid spectrum, as well as ECG examinations, echocardiography and coronary angiography, and, if necessary, computer or magnetic resonance imaging.

Ultrastructural changes in cells of venous blood of patients were studied using electron microscopy. For this purpose, 9 ml of blood was taken from the ulnar vein of patients on an empty stomach into a siliconized test tube, which was mixed with a 2% solution of sodium citrate in a ratio of 9:1 and centrifuged for 10 minutes at 150 G until the sediment of the main mass of blood cells was formed. The supernatant part of the blood was transferred to a clean test tube and centrifuged for another 10 minutes at 300 G to obtain a sediment of blood cells, including erythrocytes, leukocytes and platelets. The obtained material was washed with cacodylate buffer and transferred to a 1% OsO4 solution for 60 min to fix them. After that, the sediment from these blood cells was washed in cacodylate buffer, followed by its dehydration in alcohol solutions of increasing concentration (30, 50, 70, 90%), as well as in 100% acetone for 10 min. Further, the obtained «film» with fixed blood cells was placed in a mixture of epon and araldite, which was polymerized in gelatin capsules at a temperature of 60ºC. Ultrathin sections were prepared on a UMTP-3 ultramicrotome using diamond knives. Sections of blood cells were contrasted in lead citrate according to the method of E. Reynolds before the research [29]. Contrasted sections were examined and photographed using an electron microscope PEM100-01 at a magnification of 2,000 to 50,000 times.

When performing the work, the basic requirements of bioethics regarding the conduct of scientific medical research with the participation of a person as the object of research were observed, and the voluntary informed consent of the patients was signed. The results of the study were considered at the meeting of the committee on the ethics of scientific research, experimental developments and scientific works of the Danylo Halytsky Lviv National Medical University (protocol No. 8 dated October 21, 2019). All moral and ethical norms in accordance with the principles of the Declaration of Helsinki, the Council of Europe Convention on Human Rights and Biomedicine, ICH GCP and the current regulatory and legal acts of Ukraine were observed.

3. RESULTS

In the presence of COVID-19, we found both calcification and destruction of erythrocytes and platelets, which we did not observe in patients without COVID-19 (Fig. **1**).

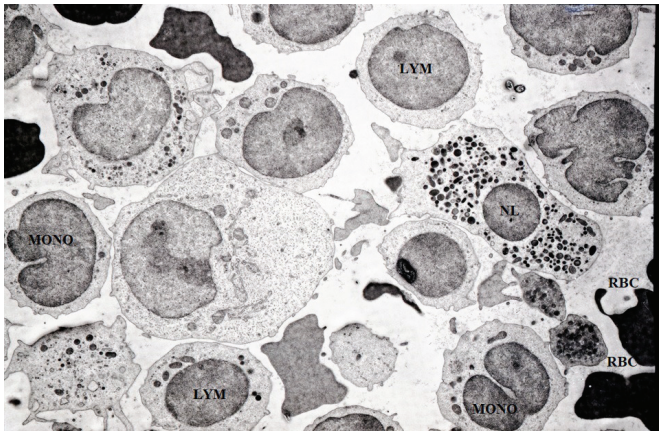

Fig. (1). Ultrastructure of venous blood cells in a patient with ischemic heart disease. Among them are neutrophil leukocyte (NL), lymphocyte (LYM), monocyte (MONO), as well as moderately calcified and deformed erythrocytes (RBC). A control study. Zoom x 7000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Calcification of erythrocytes, platelets, and sometimes leukocytes was observed in 10 patients from the comparison group who suffered acute myocardial infarction and did not suffer from COVID-19. The percentage of affected erythrocyte cells depended on the severity and stage of the disease and was 5-40% (per one hundred RBC). Reticulocytes were detected much more often in these individuals than in the comparison group (Fig. **2**).

In patients who have acute myocardial infarction with ST-segment elevation in the presence of type 2 diabetes mellitus and COVID-19, in addition to pronounced osmophilia, a significant number of significantly deformed, hemolyzed erythrocytes or with signs of acanthosis, which stuck together and with other destructively changed blood cells, was found. The blood plasma, among which there were erythrocytes, was also excessively osmiophilic, which arose as a result of hemolysis of erythrocytes. Therefore, a significant number of erythrocytes was hemolyzed, and under these circumstances, only their «shadows» were often observed,

that is, formations of an elongated snake-like shape with thinning and dumbbell-like expansion at the ends (Fig. **3**).

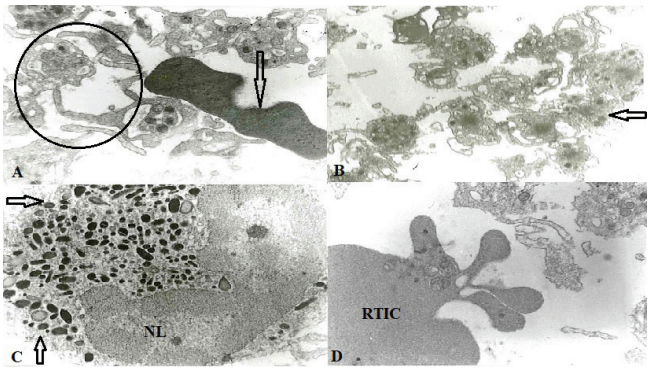

Fig. (2). Patient S., 40 years old, COVID-19, mild course, outpatient treatment: (**A**) A "bitten" erythrocyte (→), surrounded by degranulated platelets (O); (**B**) Degranulated platelets (→); (**C**) Neutrophilic leukocyte (NL) with signs of lysis of the outer cytoplasmic membrane (→); (**D**) Reticulocyte (RTIC) and necrotically changed platelets. Zoom x 3000-12000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

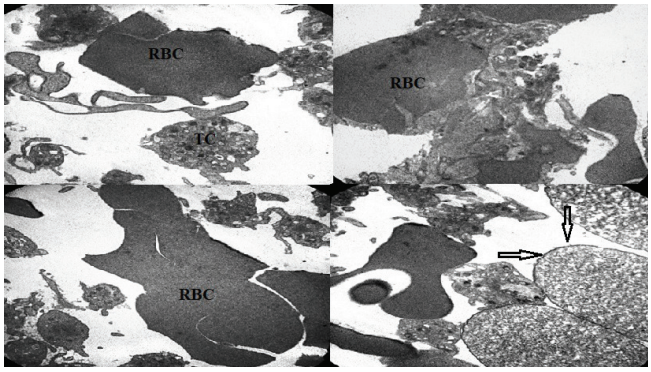

Fig. (3). Destructive changes in blood cells characteristic of COVID-19. Patient I., 58 years old. Ischemic heart disease. Acute myocardial infarction. Hypertensive disease stage III, degree 2, the risk is very high. Type II diabetes.Obesity. COVID-19, moderate severity. Calcified, deformed and fragmented erythrocytes (RBC) with signs of acanthosis and fragmentation predominate. Some of them are hemolyzed (→). Thrombocyte (TC). Zoom x 5000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

In cases of fragmentation of some erythrocytes, «balls» were formed, apparently covered with osmiophilic fibrin. In addition, we detected neutrophil extracellular traps, which can be the cause of embolization of vessels of most vital organs of the human body (Fig. **4**).

Disk-shaped and mushroom-shaped erythrocytes with pointed edges in the absence or presence of local cytoplasmic defects, dumbbell-shaped and hemolyzed cells, as well as reticulocytes were also detected. An unusual phenomenon was the presence of spherical and sometimes elongated erythrocytes, which were in contact with each other and had slightly larger sizes than usual due to cytoplasmic swelling (Fig. **5**).

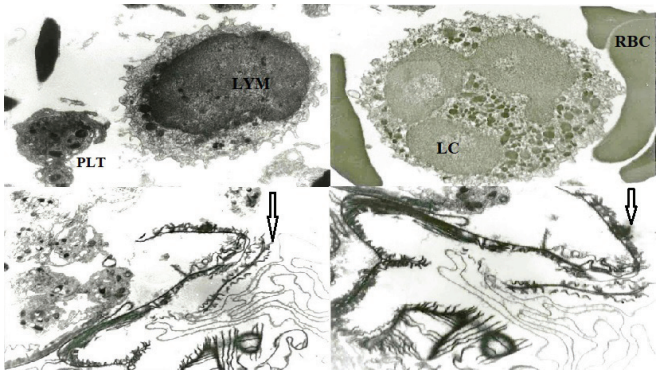

Fig**. (4).** Degenerative changes in blood cells characteristic of COVID-19. Patient U., 67 years old. Bilateral pneumonia. Ischemic heart disease. Acute myocardial infarction. Intramural clot in LV. Hypertensive disease, stage III. Type II diabetes. Atrial fibrillation. Obesity. Ultrastructure of extracellular neutrophil traps (→), next to which are calcified platelet (PLT), necrotically changed lymphocyte (LYM) and neutrophilic polymorphonuclear lymphocyte (LC) and erythrocytes (RBC). Zoom x 5000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

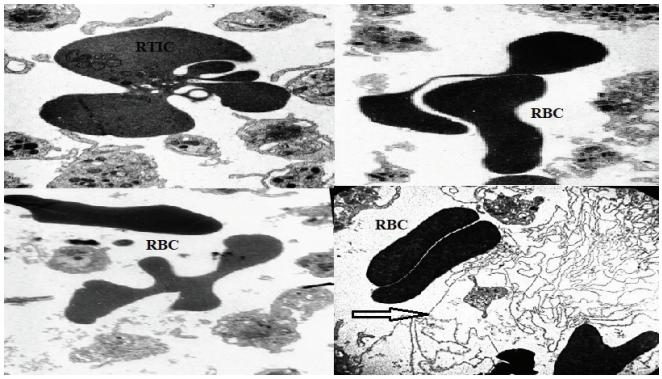

Fig. (5). Degenerative changes in blood cells characteristic of COVID-19. Extracellular neutrophil traps (→). Zoom x 5000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

However, the latter had a coarse-grained texture with lightening, which was the result of uneven condensation of calcified hemoglobin. In the osmiophilic cytoplasm of the blood, it was possible to observe a large number of significantly damaged platelets with signs of calcification (osmiophilia) and manifestations of apoptosis, which was evidenced by microclasmatosis of numerous microvilli and cytoplasmic protrusions. Platelet necrosis was also observed.

The described damage and destruction of erythrocytes were the cause of significant osmophilia of the blood plasma and worst of all, the destroyed erythrocytes lost hemoglobin, and therefore, the ability to capture O_2 in the lungs and deliver it to all vital organs of the human body, and, accordingly, to eliminate CO_2 (Fig. **6**).

The capillaries of the lungs are obstructed by fragments of other blood cells and fibrin filaments, which leads to acute respiratory failure, cytokine storm, as well as embolization

of the microcirculatory channel of most parenchymal organs of the human body (Fig. **7**).

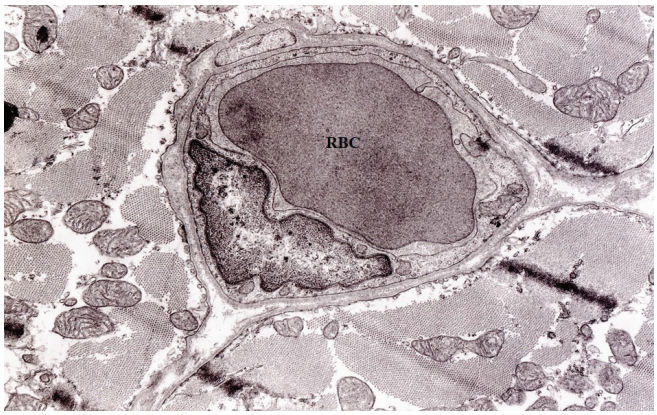

Fig. (6). Control study. In the lumen of the blood capillary, there is an erythrocyte (RBC), which behaves like a drop of liquid, adapting to the topography of the microvessels. Electron micrograph. Zoom x 8000. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

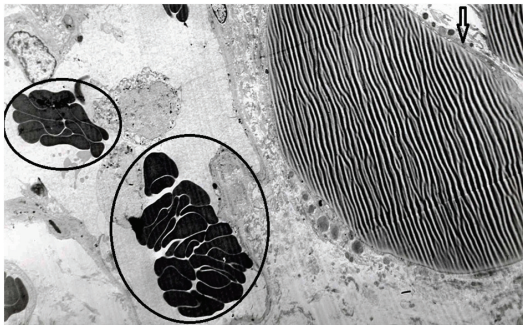

Fig. (7). Dilated venule. Stasis of calcified blood erythrocytes (O) as a factor of high risk of unfavorable course of COVID-19. Fat drop of large sizes (→) in the interstitium. Acute myocardial infarction, intact area. Patient S., 79 years old. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

Under physiological conditions, in a healthy person, erythrocytes make up 96% of all blood cells, leukocytes – 3%, and platelets – approximately 1%. In healthy individuals, erythrocytes are mostly shaped like a biconcave disc and do not contain a nucleus. The diameter of the erythrocyte is 8 microns, but the peculiarities of the cell structure and the membrane structure ensure their great ability to deform and pass through capillaries with a narrow lumen of 2-3 microns (Fig. **8**) [30].

This ability to deform is provided due to the interaction between the proteins of the membrane and the cytoplasm, while the defects of these proteins lead to morphological and functional disorders of erythrocytes. The main functions of erythrocytes are determined by the presence of the hemoglobin protein in their cytoplasm, due to which respiratory, regulatory, transport, protective functions are carried out, as well as participation in the blood clotting process. It is known that the lifespan of erythrocytes is 120 days, leukocytes – less than 1 day, platelets – 10 days, monocytes – 12 hours [30].

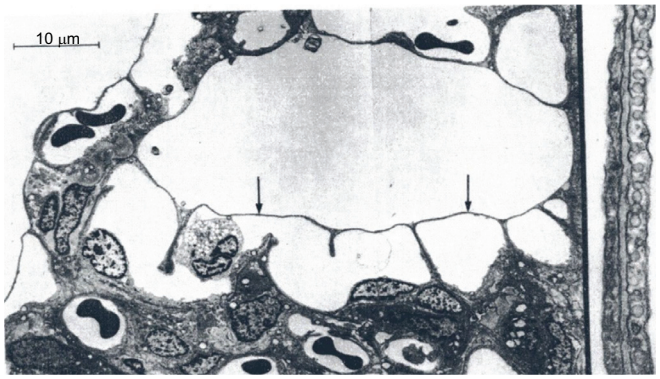

Fig. (8). Histological structure of alveolar septa. Erythrocytes block the narrow lumen of the capillaries located in the alveolar septa of the lungs. "Color Atlas of Microscopic Anatomy" Frihjof Hammersen M.D. Munich 1985. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The erythrocytes color under physiological conditions is due to the presence of a red respiratory pigment – hemoglobin, which as a protein component contains globin (consists of two α- and two β-chains), while the non-protein component is heme, which contains iron (Fe^{2+}). In the lungs, the hemoglobin of erythrocytes upon contact with oxygen is transformed into a relatively unstable compound – oxyhemoglobin. In turn, oxyhemoglobin, which supplies all cells and tissues of the human body with oxygen, is further recovered to deoxyhemoglobin [31].

The shape and elasticity of erythrocytes significantly depends on Ca^{2+}: an increase in the content of intracellular Ca^{2+} leads to changes in their shape and volume, an expand in cell stiffness and tendency to hemolysis [31]. Apoptosis processes in erythrocytes (eryptosis) can be caused by energy deficit, osmotic shock, or oxidative stress [32]. Energy deficit (decrease in the activity of the Ca^{2+}-ATPase pump) leads to the entry of calcium into the cells, which, in turn, accelerates the transmembrane movement of potassium and chloride with further dehydration of the cell. Energy deficit, together with a decrease in glutathione reserves, also weakens the antioxidant protection of erythrocytes, which similarly activates transmembrane cation channels, increasing the flow of calcium into the cell. Atherogenic and biochemical changes in the blood in IHD, namely, violation of lipid metabolism with further activation of lipid peroxidation, hyperoxidation and oxidative stress with the formation of free radicals [33] lead to the transition of oxyhemoglobin to methemoglobin, increased activity of proteases, caspases, externalization of phosphatidylserine, increased content intracellular calcium, and later – to the initiation of apoptosis processes and disruption of the structure and function of erythrocytes [34].

According to the results of our research, eryptosis is largely caused by calcification of the erythrocyte membrane, which, in turn, reduces its elasticity and rheological properties. Therefore, «rigid» erythrocytes are unable to penetrate

the capillaries of the alveoli of the lungs and myocardium, which leads to microcirculation disorders. Under these circumstances, hypoperfusion and ischemic changes occur in the vital organs of the human body. As for the elderly, their chillines are also caused by microcirculation disorders in the lower extremities. As for shortness of breath and memory weakening in the elderly, microcirculation disorders in the human body are caused by calcification of erythrocytes, and not only by atherosclerotic changes in arteries and arterioles [35].

It has been established that the intensity of apoptosis processes are affected by many endogenous physiological factors, in particular, nitric oxide, reactive oxygen species, increased concentration of ions (Ca^{2+}andMg^{2+}), and adenine nucleotides (ADP, ATP) [36]. It is known that calcium ions have a direct effect on the apoptosis of cardiomyocytes in IHD [37]. An increase in the concentration of the cytosol of cells can be due to excessive penetration of Ca^{2+} from the intercellular space through the plasma membrane and as a result of disruption of intracellular homeostasis caused by dysfunction of mitochondria and endoplasmic reticulum. An increase in intracellular Ca^{2+} under these circumstances leads to apoptosis processes, which gives reason to consider it an important marker and one of the initiators of eryptosis [38].

We detected that defeats of erythrocytes explain the cause of acute respiratory failure in patients with COVID-19. In addition, it is known that hemoglobin must remove CO_2 from the body through the lungs. But even this vital function of erythrocytes suffers significantly. Another consequence of blood plasma calcification is that, as is known, under these circumstances hypercoagulation occurs, which causes blood circulation disorders in all vital organs of the human body as a result of the appearance of «red» blood clots resistant to thrombolysis. Finally, under pointed circumstances, such a classic syndrome characteristic of COVID-19 as severe respiratory failure occurs due to the fact that calcified erythrocytes clog the capillaries of the alveolar partitions of the lungs.

It is known that the COVID-19 virus contains so-called non-structural proteins (orflab, orf3a, orf8), which have the ability to attack the β_1-chains of hemoproteins, in particular hemoglobin, dragging thus porphyrin and inhibiting heme metabolism [12, 39]. Therefore, there is a dissociation of iron atoms contained in erythrocytes. Under these conditions, iron atoms accumulate in cells, causing toxic oxidative stress and destroying them, entering the tissues of vital organs [40, 41]. The rigidity of erythrocytes is due to the loss of their elasticity due to Fe^{2+} saturation, so they cannot penetrate the capillaries of the lungs. On the other hand, such rigid erythrocytes are not saturated with O_2 and lose the ability to deliver it to all vital organs and cells of the human body. Thus, the elimination of CO_2 from the human body is disrupted, which leads to blood acidosis.

Under the influence of the acute SARS-Cov-2 infection, platelets release extracellular vesicles together with the increase in the number of platelet-leukocyte aggregates. Prolonged hypoxia and associated acidosis trigger the erythrocytes to activate platelet aggregation and thrombosis through direct interaction with the platelets or indirectly through releasing chemical signaling [42]. Platelet aggregation and macroplatelets in COVID-19 patients reflect platelet hyperactivity [43]. Activated platelets interact with neutrophils, promoting the process of NETosis, and with a dysfunctional endothelium inducing prothrombotic features [44]. Neutrophils from COVID-19 patients show decreased granularity, which suggests a pre-activated state, and increased ability to spontaneously form NETs, which contribute to the inflammatory storm that leads to respiratory failure in many patients with COVID-19 [45].

CONCLUSION

Morphological changes of blood cells in COVID-19 varied according to the disease course and severity especially in the background of a weakened immune system in older and elderly people, in the presence of diabetes, excessive body weight, cardiovascular diseases and occupational hazards. Under the influence of COVID-19, blood cells are destroyed by apoptosis and necrosis. Therefore, hypoxia and ischemia of vital organs of the human body occur. Anisocytosis of erythrocytes and large platelets are found in patients with severe COVID-19, these changes may help to identify patients with a higher risk of severe course of SARS-CoV-2 infection.

LIST OF ABBREVIATIONS

ADP	=	Adenosine Diphosphate
ATP	=	Adenosine Triphosphate
Ca^{2+}	=	Calcium
Ca^{2+}-ATPase	=	Calcium Adenosine Triphosphatase
CK-MB	=	Creatine Phosphokinase-MB
COVID-19	=	Coronavirus Disease 2019
CO_2	=	Carbon Dioxide
ECG	=	Electrocardiogram
Fe^{2+}	=	Iron
ICH GCP	=	Good Clinical Practice
IHD	=	Ischemic Heart Disease
LYM	=	Lymphocyte
Mg^{2+}	=	Magnesium
MONO	=	Monocyte
NETs	=	Neutrophils Extracellular Traps
NL	=	Neutrophil Leukocyte
OsO_4	=	Osmium Tetroxide
PLT	=	Platelet
RBC	=	Erythrocyte
RTIC	=	Reticulocyte
SARS-CoV-2	=	Severe Acute Respiratory Syndrome Coronavirus 2

TC	=	Thrombocyte
UMTP-3	=	Ultramicrotome
O_2	=	Oxygen

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was considered at the meeting of the committee on the ethics of scientific research, experimental developments and scientific works of the Danylo Halytsky Lviv National Medical University (protocol No. 8 dated October 21, 2019).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or research committees and with the 1975 Declaration of Helsinki, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

STANDARDS OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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