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ABSTRACT

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FEATURES OF ULTRASTRUCTURAL CHANGES IN THE LUNGS IN SEVERE COURSE OF COVID-19 INFECTION

Introduction. An essential point in the pathogenesis of COVID-19 is endothelial dysfunction with the development of thrombosis and microangiopathy of pulmonary vessels, which is one of the causes of high mortality. At the same time, electron microscopic examination of the pulmonary vascular bed in COVID-19 coronavirus infection is rarely performed.

Objective: To investigate ultrastructural changes in the pulmonary microcirculatory bed by determining the features of endothelial damage and the role of vascular disorders in the pathogenesis of severe COVID-19 coronavirus infection.

Methods. The material was collected at autopsy, no later than 2 hours after the fact of death of patients, fixed in Millonig's fixative with pH 7.36. Dehydration was carried out in increasing-strength ethanol, transferred to propylene oxide, and tarred in a mixture of Araldite. Ultrathin sections with a thickness of 60 nμ were made using an LKB 2188 Ultrotome NOVA ultramicrotome. According to Reynolds, sections were mounted on support grids and contrasted with uranyl acetate and lead citrate. The obtained samples were viewed in a transmission electron microscope TEM 100-01, and photofixation was carried out using a KAPPA Image Base digital camera.

Results. Significant structural changes in type 2 pneumocytes were observed with the development of degeneration and reactive hyperplasia, the formation of syncytial elements, dyscirculatory disorders with endothelial alteration, pronounced hyperemia and stasis, coagulopathy, and thrombosis. In the lumen of the alveoli, in addition to the deposition of fine-grained masses of fibrin hyaline membranes, fibrinous exudate, desquamated type 2 pneumocytes, macrophages, lymphocytes, plasma cells, single neutrophils, and erythrocytes were detected.

Individual type 2 pneumocytes were characterized by the appearance of “giant lamellar bodies” measuring 2-4 μm, which occupied a

significant part of the cytoplasm. Hyperplasia of type 2 pneumocytes was observed in some areas of the lung tissue. The proliferation of fibroblasts and collagen fibrils was detected in the interstitium of the interalveolar septa.

Conclusions. As a result of transmission electron microscopy of the lungs of patients who died due to severe COVID-19 coronavirus infection, pronounced dyscirculatory changes were found in the vessels of the microcirculatory bed, characterized by the development of hyperemia, stasis, and microthrombosis with pronounced degenerative, necrotic changes in the endothelium and the development of endotheliitis.

Keywords: COVID-19 coronavirus infection, coronavirus pneumonia, lungs microcirculatory bed, coronavirus endoteliitis, transmission electron microscopy.

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ОСОБЛИВОСТІ УЛЬТРАСТРУКТУРНИХ ЗМІН ЛЕГЕНЬ ПРИ ВАЖКОМУ ПЕРЕБІГУ КОРОНАВІРУСНОЇ ІНФЕКЦІЇ COVID-19

Вступ. Важливою ланкою патогенезу COVID-19 вважають ендотеліальну дисфункцію з розвитком тромбозу та мікроангіопатію судин легень, що є однією із причин високої летальності. В той же час, електронно-мікроскопічне дослідження легеневого судинного русла при коронавірусній інфекції COVID-19 проводилося рідко.

Мета: дослідження ультраструктурних змін мікроциркуляторного русла легень із визначенням особливостей ураження ендотелію та ролі судинних розладів у патогенезі важкого перебігу коронавірусної інфекції COVID-19.

Методи. Матеріал відбирали автопсійно, не пізніше 2 годин після встановлення факту смерті пацієнтів, фіксували у фіксаторі Міллоніга з pH 7,36. Дегідратацію здійснювали в етанолі зростаючої міцності, переносили в пропілен-оксиду і просмолювали в суміші аралдіту. Ультратонкі зрізи товщиною 60 nμ виготовляли за допомогою ультрамікротома LKB 2188 Ultrotome NOVA. Зрізи монтували на опорні сітки, контрастували ураніл-ацетатом і цитратом свинцю по Рейнольдсу. Отримані зразки переглядали в трансмісійному електронному мікроскопі ПЭМ 100-01, фотофіксацію здійснювали за допомогою цифрової фотокамери KAPPA Image Base.

Результати. Спостерігалися значні структурні зміни пневмоцитів 2 типу із розвитком дегенерацією та реактивною гіперплазію, формуванням синцитіальних елементів, та дисциркуляторними розладами з альтерацією ендотелію, вираженою гіперемією та стазом, коагулопатією та тромбозом. У просвіті альвеол, окрім відкладання дрібнозернистих мас фібринових гіалінових мембран, виявлено фібринозний ексудат, десквамовані пневмоцити 2 типу, макрофаги, лімфоцити, плазматичні клітини, поодинокі нейтрофіли та еритроцити.

Окремі пневмоцити 2 типу характеризувалися появою “гігантських ламелярних тілець” розміром 2-4 мкм, які займали значну частину цитоплазми. В окремих ділянках легеневої тканини спостерігалася гіперплазія пневмоцитів 2 типу. В інтерстиції міжальвеолярних перетинок виявлено проліферацію фібробластів та фібрил колагенових волокон.

Висновки. У результаті проведення трансмісійної електронної мікроскопії легень пацієнтів, які померли внаслідок важкого перебігу коронавірусної інфекції COVID-19, в судинах мікроциркуляторного русла виявили виражені дисциркуляторні зміни, що характеризуються розвитком гіперемії, стазу та мікротромбозу з вираженими дегенеративними, некротичними змінами ендотелію та розвитком ендотеліїту.

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INTRODUCTION / ВСТУП

Despite the fact that four years have passed since COVID-19 was declared a global SARS-CoV-2 pandemic in March 2020 [1, 2] and that today the incidence has significantly decreased, some questions about the pathogenesis of COVID-19 remain unsolved. It is known that COVID-19 was first detected in December 2019 in Wuhan (China) after a series of pneumonias of unknown etiology. As of March 19 (2020), more than 225,000 cases of COVID-19 were reported in over 160 countries, resulting in over 9,200 deaths and 84,000 recovery cases. The situation was recognized as a pandemic by the World Health Organization. In March 2020, the World Health Organization (WHO) declared COVID-19 a pandemic, and pharma companies began developing vaccines. In total, about 704 million cases of COVID-19 were confirmed worldwide during the epidemic, and more than 7.01 million people died. In Ukraine, 5.5 million cases and 112,480 deaths were registered during the epidemic [1–5].

The SARS-CoV-2 virus was shown to use angiotensin converting enzyme 2 (ACE2) receptors to infect cells. ACE2 protein is expressed in significant amounts in various cells of the human body, in particular the lungs, heart and blood vessels, gastrointestinal tract, and urinary system [6, 7]. The glycoprotein trimer, through which the coronavirus interacts with the cell, consists of two subunits: S1 interacts with receptor molecules and S2 is responsible for the fusion between the viral envelope and the host cell [8, 9]. Virus-infected cells undergo alternative changes, with subsequent activation of alveolar macrophages, production of pro-inflammatory cytokines and chemokines, and development of a “cytokine storm” [10, 11].

An important link in the pathogenesis of COVID-19 is considered to be thrombosis and microangiopathy of the pulmonary vessels, which is the result of endothelial dysfunction [12–14]. It is worth noting that electron microscopic examination of the pulmonary vascular bed in patients with COVID-19 infection was rarely performed. In this regard, the study of ultrastructural changes in the pulmonary microcirculatory bed will allow us to determine the features of endothelial damage and establish the role of vascular disorders in the pathogenesis of severe COVID-19 infection.

MATERIALS AND METHODS

The material was collected at autopsy using a puncture needle, no later than 2 hours after the patient’s death was established. The study group consisted of ten people, aged 34 to 85 years, with a male to female ratio of 1 to 1.5. The duration of the disease ranged from 9 to 40 days (1 patient died on day 9, three patients on day 14, three patients on day 17, and one patient each on days 18, 22, and 40). The material was fixed in Millonig's fixative with pH 7.36. For this purpose, Millonig phosphate buffer 0.2 M was combined with a 1.4% osmium tetroxide solution in a 1:1 ratio. As a result, a 2% solution of osmium tetroxide in 0.1 M Millonig phosphate buffer was obtained. Dehydration of the material was carried out in ethanol of increasing concentration in the range from 10% to 70% ethanol solution in distilled water. Further, the material was kept in 3 portions of absolute ethanol for 10 min each, transferred to 2 portions of propylene oxide for 5 min each and embedded for 24 h in a mixture of araldite of the following composition: Araldite M, hardener HY 964 (1:1), and thoroughly mixed. Ultrathin sections with a thickness of 60 nμ were obtained using LKB 2188 Ultrotome NOVA ultramicrotome. The obtained

sections with a thickness of 60 nμ were mounted on support grids through water, dried for 2 h at a temperature of 60 °C and stained with uranyl acetate and Reynold's lead citrate. Then, the matertial was washed in 0.02 M NaOH solution, and then in distilled water, followed by drying. The samples were examined using a TEM 100-01 transmission electron microscope, and photofixation was performed using a KAPPA Image Base digital camera. The electron microscopy findings were evaluated by two independent pathologists. This study was approved at the Meeting No. 5 of the Bioethics Committee of the Danylo Halytsky Lviv National Medical University, dated February 11, 2023. The relatives of the deceased provided their informed consent for lung tissue collection.

RESULTS

Ultrastructural examination of the respiratory tract in five patients who died during their proliferative stage (day 17–22 of the disease) revealed alteration and reactive hyperplasia of type 2 pneumocytes with the formation of syncytial elements, pronounced alteration of the endothelium, coagulopathy and thrombosis of vessels of various calibers, and the induction of fibrotic processes.

Degenerative changes in type 2 pneumocytes were characterized by significant damage to apical microvilli, structural components of the endoplasmic reticulum, and mitochondria. In addition to the heterogeneous expansion of the tubules of the granular endoplasmic reticulum, mitochondrial swelling and mitochondrial cristae destruction; some of the type 2 pneumocytes developed “giant lamellar bodies” of 2-4 μm, which occupied a rather significant volume of the cytoplasm. “Giant lamellar bodies” often contained heterogeneous electron-dense masses in the center. In addition, deformed and loose lamellar contents in lamellar bodies were noted (Fig. 1).

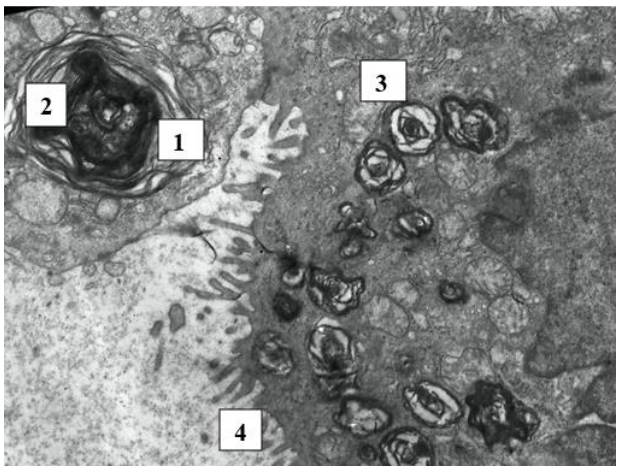

Figure 1 – Degenerative changes in type 2 pneumocytes: giant lamellar body in the cytoplasm of type 2 pneumocytes (1) with heterogeneous electron-dense material in the center (2) and loose lamellar contents (3); destruction of apical microvilli (4). Electron diffraction pattern x 6000

Due to the pronounced destruction of apical microvilli on some free surface of type 2 alveolocytes, microvilli were not visualized or only single small remnants of apical microvilli were observed (Fig. 2). In desquamated type 2 pneumocytes, destruction of the cytoplasmic membrane was noted in addition to damaged mitochondria and apical microvilli, and chaotic arrangement of the lamellar contents in the lamellar bodies.

Fine-grained, structureless masses of hyaline membranes were found in the lumens of the alveoli. The alveoli revealed exudate containing filamentous masses of fibrin and desquamated type 2 pneumocytes, and an inflammatory macrophage-lymphocyte infiltrate with admixtures of neutrophils. Single fibroblasts were identified in the stroma of the alveolar septa.

It is worth noting that some areas of the lung tissue were clearly hypercellular due to the hyperplasia of type 2 pneumocytes, as well as the presence of an excessive number of fibroblastic interstitial cells and collagen fibrils in the connective tissue of the interalveolar septa. Inflammatory infiltration of the interstitium by lymphocytes, plasma cells, macrophages, and isolated neutrophils was observed. Hyperplastic type 2 pneumocytes were tightly packed together, their nuclei contained several nucleoli and an excessive amount of heterochromatin; sometimes they had an irregular rounded shape, and destruction of apical microvilli was observed. In hyperplastic type 2 pneumocytes, we noted heterogeneous expansion of the tubules of the granular

endoplasmic reticulum, mitochondrial swelling and mitochondrial cristae destruction (Fig. 3). We found type 2 pneumocytes with a pyknotic nucleus and pronounced destruction of cytoplasmic organelles. The giant multinucleated cells occurred as a reaction to a pronounced respiratory epithelium alteration during the exudative stage, including viral damage to pneumocytes. Hypercellularity of the respiratory tract is caused not only by hyperplasia of type 2 pneumocytes, but also by infiltration of the interstitium by neutrophils, macrophages, single lymphocytes, and accumulation of active fibroblasts.

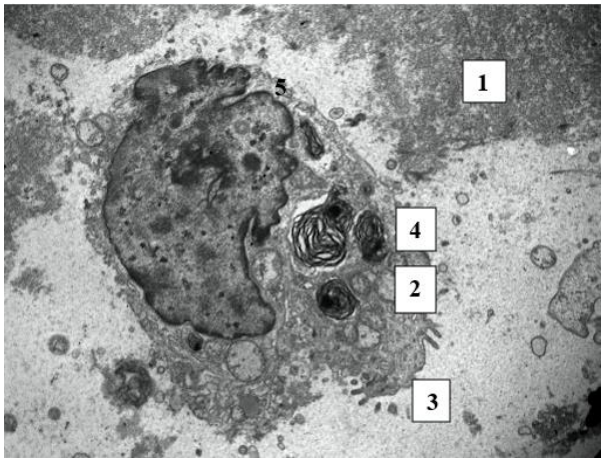

Figure 2 – Degenerative changes in type 2 pneumocytes: fine-grained masses of hyaline membrane (1) and a desquamated type 2 pneumocyte (2) in the lumen of the alveolus; destruction of apical microvilli of a type 2 pneumocyte (3); swelling of mitochondria and destruction of mitochondrial cristae with chaotic arrangement of lamellar contents (4); destruction of the cytoplasmic membrane (5). Electron diffraction pattern x 3800

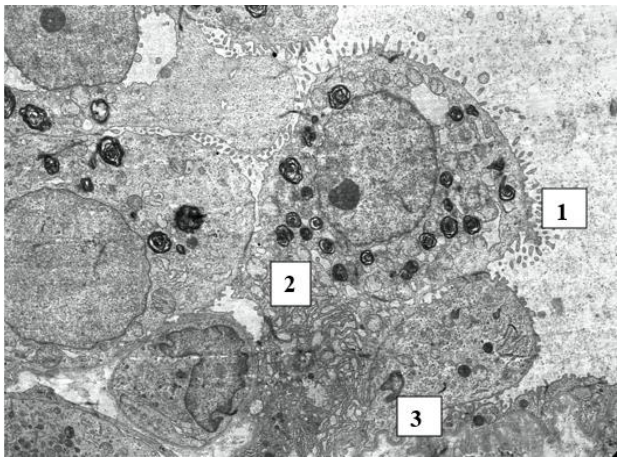

Figure 3 – Hyperplasia of type 2 pneumocytes (1); destruction and desquamation of apical microvilli of type 2 pneumocytes, destruction of mitochondrial cristae (2); pyknotic nucleus of a type 2 pneumocyte (3). Electron diffraction pattern x 2200

In some areas of the lung tissue, giant multinucleated cells appeared, the nuclei of which contained intensely osmiophilic nucleoli. Sometimes atypical proliferating pneumocytes formed syncytium. The alveolar-capillary basement membrane was mostly heterogeneously thickened, luminal, and sometimes acquired low electron density.

Pronounced dyscirculatory changes were observed in the vessels of the pulmonary microcirculatory bed. Due to severe hyperemia, venules and arterioles were significantly dilated and crowded with erythrocytes, but in some places they contained neutrophils. Often, erythrocytes were of heterogeneous shape and swollen, and deep masses of fibrin were often visualized next to them (Fig. 4). Condensed masses of blood plasma mixed with fibrin accumulated in some vessels, and platelets were visualized in some places. There were single lymphocytes that were adjacent to the luminal surface of endothelial cells. In addition, adhesion of neutrophils and erythrocytes to the luminal surface of endothelial cells was observed. Increased number of cytoplasmic granules was noted in the cytoplasm of neutrophils, and vacuolization of the cytoplasm was observed in individual neutrophils.

The lumens of most capillaries were completely filled with swollen erythrocytes forming a sludge. Single lymphocytes and neutrophils were detected in some capillaries.

Pronounced alterative changes with the development of focal necrosis were observed in the endothelium; they were characterized by sharp clearing of the hyaloplasm and pronounced destruction of organelles. The nucleus was characterized by pyknotic changes with chromatin condensation, while the karyolemma lost its clear contours. Often, the basement membrane of some capillaries lacked its electron density. In areas of severe endothelial alteration, exposure of the basement membrane of the microcirculatory vessels was noted and thrombi formation out of the conglomerates of platelets, erythrocytes, neutrophils, and fibrin was observed.

Degenerative changes in the endothelium were characterized by heterogeneous expansion of the tubules of the granular endoplasmic reticulum and destruction of ribosomes. Swelling of mitochondria in the endothelial cytoplasm, clarification of their matrix, and destruction of cristae were observed. Medium electron density lipoproteins accumulated in the cytoplasm of individual endothelial cells.

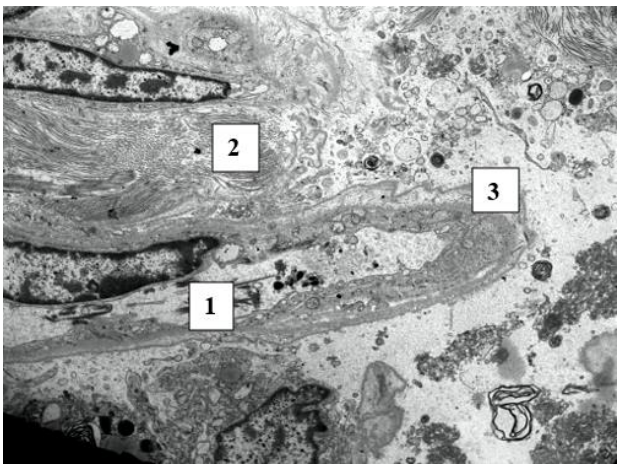

Figure 4 – Fibrin strands in the lumen of the venule (1); a significant number of collagen fibrils in the stroma (2); perivascular edema (3). Electron diffraction pattern x 2200

Numerous virion-like elements were visualized in the cytoplasm of individual endothelial cells, with an electron-dense nucleus and spike-like masses on the periphery, which were quite often limited by a double-contour membrane.

The described lesions of hemocapillaries, in particular degenerative and necrotic changes in the endothelium, with the presence of virion-like inclusions in the cytoplasm, and the localization of lymphocytes and neutrophils in the lumen of the vessels, which were in contact with endothelial cells, indicated the development of endotheliitis in patients with COVID-19.

In addition, as a result of alterative changes in the endothelium, vascular permeability increased, which was accompanied by the exit of single erythrocytes outside the vessels and their accumulation in the interstitium. In addition to erythrocytes, an inflammatory infiltrate was observed in the connective tissue of the interalveolar septa, consisting mainly of macrophages, single plasma cells, lymphocytes, and neutrophils. The interstitial fibrosis processes were noted, characterized by increased number of active fibroblasts and numerous bundles of collagen fiber fibrils.

DISCUSSION

The pathomorphology of pulmonary vascular lesions is one of the fundamental elements in the study of the morphogenesis of COVID-19 infection. It is known that the SARS-CoV-2 has a cytopathic effect on type 2 pneumocytes, cardiomyocytes, epithelial cells of the respiratory tract, urinary system organs, neurons, and, importantly, has endotheliotropic properties [14, 15]. Endothelial cell damage occurs as a result of virus penetration into endothelial cells, damage to cell membranes, with the subsequent development of microangiopathy and thrombosis. Along with the direct cytopathic effect of the virus, important elements of the COVID-19 infection pathogenesis are uncontrolled immune and inflammatory responses, as well as vascular homeostasis impairment [16]. The endothelium plays a crucial role in the regulation of vascular tone, synthesizing substances that have both vasoconstrictor and vasodilator effects [17]. Endothelial dysfunction is a basic determinant of the development of dyscirculatory processes in COVID-19, which are characterized by vasoconstriction, ischemia, and a procoagulative state of the blood [18].

The complications most commonly observed in deceased patients with COVID-19 were usually associated with endothelial damage and severe dysfunction of the blood coagulation system, the development of a wide range of coagulopathies, which were accompanied by high levels of D-dimer and plasma fibrinogen [19].

Thrombi caused by SARS-CoV-2 coronavirus infection can occur in vessels of various calibers, both in the arterial and venous system [20]. Acute lung injury due to COVID-19 infection is accompanied by activation of macrophages, lymphocytes, and neutrophils. Macrophages synthesize cytokines and chemokines, including monocyte chemotactic protein 1 (MCP-1), interferon-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1, and release these mediators into the alveolar lumen. Subsequently, the complement system and fibrinolytic system are activated, endothelium alters, vascular permeability increases, and thrombus formation occurs followed by the release of a significant number of fibrin degradation fragments (d-dimers) into the bloodstream [21].

In the course of our ultrastructural study of the hemomicrocirculatory bed vessels of the lungs in patients with COVID-19, we detected pronounced dyscirculatory findings characterized by a change in vascular balance towards vasoconstriction and the development of hypercoagulability. It is worth noting that accumulations

of aggregated erythrocytes, platelets, and fibrin were found in capillaries, venules, and arterioles, indicating the development of active thrombus formation in the pulmonary vessels.

Hypercoagulability in COVID-19 infection is believed to be both systemic and local in nature. Local hypercoagulability is caused by microangiopathy, while systemic hypercoagulability is associated with defects in the blood coagulation system [22].

It is worth noting that viral infection of the capillary endothelium is accompanied by a restructuring of intracellular metabolism into a pro-inflammatory mode with the subsequent development of endotheliitis. Endothelial lesion is accompanied by the release of thrombomodulin and the entry of tissue factor (tissue thromboplastin) into the microcirculation, triggering intravascular blood clotting [23]. This is confirmed by the endotheliitis we detected with accumulations of neutrophils on the lumenal surface of endothelial cells in combination with alterative-necrotic changes in endothelial cells.

According to Magro C. et al. [24], endotheliitis is the main pathological process that leads to multiple organ failure and even death in patients with COVID-19. Endotheliitis caused by SARS-CoV-2 is systemic in nature and develops both as a result of direct exposure to the virus and due to the host inflammatory response [25–27]. Given that, with significant damage, the endothelium loses its physiological property of regulating homeostasis, fibrinolysis, and antiaggregation, a complex of reactions is triggered with the formation of numerous thrombi, which leads to coagulopathy in all cases of COVID-19 disease [28].

Thus, endotheliitis with signs of impaired vascular barrier integrity contributing to a procoagulative state may explain the disproportionate hypoxemia that develops in patients with COVID-19. Vascular endothelium lesion with the development of necrotic changes leads to a change in the properties of the endothelial surface from antithrombotic to prothrombotic. As a result of the exposure of the proadhesive subendothelial surface, adhesive proteins, including fibrinogen, are involved in the processes of thrombus formation.

CONCLUSIONS

As a result of transmission electron microscopy of the lungs of patients who died due to severe COVID-19 infection, pronounced dyscirculatory changes were found in the vessels of the microcirculatory bed, characterized by the development of hyperemia, stasis, and microthrombosis with pronounced degenerative, necrotic changes in the endothelium and the development of endotheliitis. We identified alterative changes in the endothelium which led to hypercoagulation and occlusion of capillaries, small venules and arterioles, with pronounced disorders of blood circulation in the pulmonary microcirculation vessels; this, in our opinion, was the key moment in the severe course of COVID-19 infection that caused the development of severe respiratory failure and thrombotic complications.

PROSPECTS FOR FUTURE RESEARCH

Further pathomorphological studies of hemocoagulation disorders and endothelial dysfunction in severe COVID-19 infection are an important issue for adequate treatment and diagnostic tactics for infectious diseases.

AUTHOR CONTRIBUTIONS

All authors substantively contributed to the drafting of the initial and revised versions of this paper. They take full responsibility for the integrity of all aspects of the work.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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