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Lymphocyte apoptosis in patients with coronavirus infection COVID-19

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ABSTRACT

BACKGROUND: Lymphopenia in patients with coronavirus infection COVID-19 is associated with the risk of developing severe forms and unfavorable outcome. One of the reasons for the development of lymphopenia is apoptosis.

AIM: Evaluation of the severity of peripheral blood lymphocytes' apoptosis in patients with moderate and severe COVID-19.

MATERIAL AND METHODS: A total of 42 patients with COVID-19 aged 37 to 90 years were examined. They were hospitalized at the Republican Clinical Infectious Diseases Hospital named after Professor A.F. Agafonov, Kazan, from October 24, 2021 to March 1, 2022. In 13 patients, the lung lesion volume ranged from 10 to 25% (CT-1), in 20 — from 25 to 50% (CT-2), in 9 — from 50 to 75% (CT-3). Ribonucleic acid of the SARS-CoV-2 virus was isolated from the nasopharynx in 35 (83%) patients. COVID-19 was moderate in 14 patients, and severe in 28 patients. The control group consisted of 10 conditionally healthy people of the same age. Lymphocyte apoptosis was assessed by quantifying hypodiploid cells by changing the intensity of their staining with propidium iodide using flow cytometry. To determine the reliability of differences in indicators between the compared groups, the Mann–Whitney U-test was used, and when comparing percentages, the χ^2 criterion was used. The reliability of differences was established at $p < 0.05$.

RESULTS: It was found that patients with COVID-19 had significantly higher lymphocyte apoptosis activity compared to the control group. The median of the studied indicator in patients with COVID-19 was 39.3%, while in the control group it was 15.1% ($p < 0.001$). The severity of lymphocyte apoptosis correlated with the severity of the disease: the highest rates were recorded in patients with severe COVID-19 ($p=0.02$). Moreover, lymphocyte apoptosis >55% was associated with the risk of death ($p=0.03$). A moderate correlation was established between lymphocyte apoptosis rates and blood ferritin levels (Spearman coefficient $p=0.39$, $p < 0.05$).

CONCLUSION: Coronavirus infection COVID-19 was accompanied by an increase in the activity of peripheral blood lymphocyte apoptosis; the highest apoptosis rates were recorded in patients with severe COVID-19.

Keywords: coronavirus infection COVID-19; lymphocytes; apoptosis.

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Апоптоз лимфоцитов у пациентов с коронавирусной инфекцией COVID-19

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АННОТАЦИЯ

Актуальность. Лимфопения у пациентов с коронавирусной инфекцией COVID-19 ассоциируются с риском развития тяжёлых форм и неблагоприятного исхода. Одна из причин развития лимфопении — апоптоз.

Цель. Оценка выраженности апоптоза лимфоцитов периферической крови у пациентов со среднетяжёлым и тяжёлым течением COVID-19.

Материал и методы. Обследованы 42 пациента с COVID-19 в возрасте от 37 до 90 лет, госпитализированных в ГАУЗ «Республиканская клиническая инфекционная больница имени профессора А.Ф. Агафонова», г. Казань, в период с 24 октября 2021 г. по 1 марта 2022 г. У 13 пациентов объём поражения лёгких составил от 10 до 25% (КТ-1), у 20 — от 25 до 50% (КТ-2), у 9 — от 50 до 75% (КТ-3). Рибонуклеиновая кислота вируса SARS-CoV-2 из носоглотки была выделена у 35 (83%) пациентов. У 14 больных было среднетяжёлое течение COVID-19, у 28 — тяжёлое. Контрольную группу составили 10 условно здоровых людей аналогичного возраста. Оценку апоптоза лимфоцитов осуществляли на основании количественного определения гиподиплоидных клеток по изменению интенсивности их окраски пропидия йодидом с помощью проточной цитометрии. Для определения достоверности различий показателей между сравниваемыми группами применяли U-критерий Манна–Уитни, при сравнении процентных долей — критерий χ^2. Достоверность различий устанавливали при $p < 0,05$.

Результаты. Выявлено, что у пациентов с COVID-19 достоверно более высокая активность апоптоза лимфоцитов по сравнению с контрольной группой. Медиана изучаемого показателя у больных COVID-19 составила 39,3%, тогда как в контрольной группе — 15,1% ($p < 0,001$). Выраженность апоптоза лимфоцитов коррелировала с тяжестью заболевания: наиболее высокие показатели зарегистрированы у пациентов с тяжёлым течением COVID-19 ($p=0,02$). При этом апоптоз лимфоцитов >55% ассоциировался с риском летального исхода ($p=0,03$). Была установлена умеренно выраженная корреляционная связь между показателями апоптоза лимфоцитов и уровнем в крови ферритина (коэффициент Спирмена $p=0,39$, $p < 0,05$).

Вывод. Коронавирусная инфекция COVID-19 сопровождается повышением активности апоптоза лимфоцитов периферической крови; наиболее высокие показатели апоптоза зарегистрированы у пациентов с тяжёлым течением COVID-19.

Ключевые слова: коронавирусная инфекция COVID-19; лимфоциты; апоптоз.

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BACKGROUND

Immune dysregulation remarkably contributes to the development of severe coronavirus disease 2019 (COVID-19) [1]. Lung injury in COVID-19 is caused by a hyperinflammatory response characterized by elevated levels of blood pro-inflammatory cytokines, C-reactive protein, and ferritin [2]. However, significant lymphopenia is typical in COVID-19 [1], which is associated with a mortality risk [3].

Lymphocytes are adaptive immunity cells that are crucial in generating specific immune responses and eliminating pathogens. Lymphopenia in COVID-19 may be caused by lymphocyte death, excessive pro-inflammatory cytokine production, lymphopoiesis inhibition, and lymphocyte migration to the respiratory organs [4].

The mechanism of lymphocyte death in COVID-19 can occur through various pathways such as apoptosis, pyroptosis, autophagy, virus-specific $CD8^+$ T-lymphocyte-dependent cytotoxicity, and antibody-dependent cell-mediated cytotoxicity [4].

Considering the critical role of lymphopenia in the development of severe COVID-19, the degree of lymphocyte apoptosis in patients with COVID-19 should be examined to identify new pathogenetic targets for treatment.

This study aimed to evaluate the expression of apoptosis in peripheral blood lymphocytes in patients with moderate and severe COVID-19.

MATERIALS AND METHODS

Overall, 42 patients with COVID-19, aged 37–90 years, who were admitted to the Agafonov Republican Clinical Infectious Diseases Hospital from October 24, 2021 to March 1, 2022, were studied. The mean age of the patients was 67.3 ± 13.7 years.

Moderate or severe COVID-19 course was the inclusion criterion, and mild course was the exclusion criterion.

COVID-19 was diagnosed according to the interim guidelines for prevention, diagnosis, and treatment of COVID-19, version 10, dated February 8, 2021 [5]. Fourteen patients were diagnosed with moderate COVID-19 and 28 patients with severe COVID-19.

All patients underwent chest computed tomography (CT) and showed ground-glass opacities in the lungs. The degree of lung damage was 10%–25% in 13 patients (CT-1), 25%–50% in 20 patients (CT-2), and 50%–75% in 9 patients (CT-3). In 35 (83%) patients, COVID-19 diagnosis was confirmed by isolation of SARS-CoV-2 ribonucleic acid (RNA) from the nasopharynx (disease code U07.1). In the remaining 7 (17%) patients, diagnosis was based on clinical presentation (i.e., fever, cough, and signs of respiratory failure) and ground-glass opacities in the lungs seen on chest CT (disease code U07.2).

Among the 42 patients, 33 (88%) had comorbidities, the most common of which were hypertension (71%), diabetes mellitus (33%), and obesity (28%). Absolute lymphopenia ($<1 \times 10^9$/L) was observed in 18 (43%) of 48 patients . All patients required respiratory support, and six were on mechanical ventilation. In six cases, the disease led to death. Table 1 shows the patients' characteristics. The control group consisted of 10 conditionally healthy individuals of similar age ($p = 0.1$).

Hypertension was the most common comorbidity in the control group, accounting for 60% of cases. The main and control group patients were comparable in age, sex, and comorbidities (Table 1).

Flow cytometry (FACs Canto II, Becton Dickinson, USA) was used to investigate the degree of spontaneous apoptosis of peripheral blood lymphocytes in the acute phase of COVID-19 based on the number of hypodiploid cells and changes in their staining intensity using propidium iodide (Sigma Aldrich, USA). The results were evaluated after culturing lymphocytes for 96 hours in flat-bottomed 24-well plates (Corning, USA) in complete RPMI-1640 medium with the addition of L-glutamine, antibiotics (streptomycin and penicillin), and 10% fetal bovine serum (all reagents from PANECO, Moscow, Russia). Blood samples were collected once during hospitalization days 1–3 and on average on day 9 [95% confidence interval: 8–10]. Simultaneously, the degree of lymphocyte apoptosis was assessed, and peripheral blood lymphocytes were counted.

This was an observational study. Statistical analysis of the results was performed using Statistica for Windows 6.1 (Statsoft, Tulsa, OK, USA). The Shapiro–Wilk test was used to assess whether the characteristic's distribution conformed to the normal distribution law. The null hypothesis was rejected at a statistical significance level (p) of 0.05. If this condition was not met, nonparametric statistical analysis methods were used. If the distribution of a characteristic differed from normal, the median (Me) was utilized as a measure of central tendency and the interquartile range (25th and 75th percentiles) as a measure of dispersion. The Mann–Whitney U test was employed to assess reliability of differences in parameters between the groups, and the χ^2criterion was used when comparing percentages. Differences were considered significant at $p < 0.05$.

The relationship between two characteristics (r) were evaluated with the nonparametric method of Spearman's correlation analysis. Contingency tables were utilized to calculate sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratios (LR) for positive and negative results, and relative risk.

The Ethics Committee of the Republican Clinical Infectious Diseases Hospital approved this study (protocol No. 4/4, dated June 3, 2021). Written informed consent was obtained from the patients according to the guidelines approved under this protocol (Federal Law No. 323-FZ of November 21, 2011 on the Fundamentals of Health Protection of Citizens in the Russian Federation).

Table 1. Clinical and laboratory characteristics of patients with COVID-19

Parameter	Patients with COVID-19, n = 42	The control group, n = 10	Significance
Age, years, M ± SD	67,3±13,7	60,6±8,2	p=0,1
Female, n (%)	25 (59,5)	6 (60)	χ^2=0,02; p=0,8
Day of hospitalization, Me [interquartile range]	7 [5–8]	—	—
SARS-CoV-2 RNA isolation, n (%)	35 (83)	—	—
Lung injury extent, n (%): – CT-1, – CT-2, – CT-3	 13 (31) 20 (48) 9 (21)	—	—
Comorbidities, n (%): – Essential hypertension – Diabetes mellitus – Obesity – Heart arrhythmia – COPD – CKD – Cancer	33 (78,5) 30 (71) 14 (33) 12 (28) 4 (9) 3 (7) 2 (4) 2 (4)	8 (80) 6 (60) — 2 (20) 1 (10) 1 (10) — —	χ^2=0,12; p=0,7 χ^2=2,6; p=0,1 — χ^2=1,7; p=0,1 χ^2=0,05; p=0,8 χ^2=0,57; p=0,4 — —
Respiratory support, n (%): – Low-flow oxygen therapy – High-flow oxygen therapy – Mechanical ventilation	 31(74) 5 (12) 6 (14)	—	—
WBC, $\times 10^9$/L, Me [interquartile range]	7,2 [5,4–10,2]	5,2 [4,5–6,3]	0,01
Lymphocytes, $\times 10^9$/L, Me [interquartile range]	1,1 [0,6–1,5]	2,5 [2,1–3,4]	0,0002
Lymphocytes <10^9/L, count (%)	18 (43)	—	—
C-reactive protein, Me [interquartile range]	37,9 [9,1–70]	0	—
Ferritin, ng/mL, Me [interquartile range]	472 [229,8–855]	107,5 [86–121]	0,00003

Note: RNA, ribonucleic acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CT, computed tomography; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; WBC, white blood cells.

RESULTS

COVID-19 was found to be associated with increased apoptotic activity of peripheral blood lymphocytes (Figure 1). The median of the study parameters was 39.3% in patients with COVID-19 and 15.1% in controls ($p < 0.001$). Moreover, the degree of lymphocyte apoptosis correlated with disease severity; the highest rates were noted in severe COVID-19 patients (Table 2). Blood lymphocyte counts were lower in severe COVID-19 patients than in moderate COVID-19 patients; however, this difference was not significant ($p = 0.1$).

Considering the possible correlation between inflammatory response and lymphocyte apoptosis, a correlation analysis was performed to assess the degree of lymphocyte apoptosis with key inflammatory markers such as C-reactive protein and ferritin, with levels significantly higher in COVID-19 patients than in controls (Table 1). A moderate direct correlation was observed between lymphocyte apoptosis index and blood ferritin level (Spearman's coefficient $p = 0.39$, $p < 0.05$). No correlation was found between degree of apoptosis and level of C-reactive protein.

Evaluation of study parameters considering the disease outcome showed that the blood lymphocyte count was 2.1 times lower in patients who died than in those who survived (Table 3). Although lymphocyte apoptosis activity was higher in the group of patients who died, this difference was not significant.

The next step was to evaluate the performance characteristics of the diagnostic test for lymphocyte apoptosis when used for assessing death risk. Table 4 shows the results. Lymphocyte apoptosis >55% in patients with COVID-19 was associated with the risk of death ($p = 0.03$). However, a positive result of the cell apoptosis test was most probable (LR+ = 3.8). Thus, this test can be considered reliable.

DISCUSSION

Lymphopenia is a crucial parameter in determining COVID-19 severity and prognosis. Lymphopenia develops in 63% of patients with COVID-19 [6]. In the present study, 43% of patients had lymphocyte counts <1.0 × 10^9/L. Additionally, the degree of lymphopenia was 2.1 times greater in the group of patients who died.

Table 2. Blood lymphocyte counts in COVID-19 patients (median [interquartile range])

Parameter	Moderate COVID-19 (n = 14)	Severe COVID-19 (n = 28)	p
Lymphocytes $<10^9$/L	1.43 [0.97–1.90]	0.93 [0.58–1.35]	0.1
Apoptosis of lymphocytes (%)	28.8 [20.6–42.4]	40.8 [32.4–49.4]	0.02

Table 3. Blood lymphocyte counts in surviving and deceased patients (median [interquartile range])

Parameters	Surviving patients (n = 36)	Patients who died (n = 6)	p
Lymphocytes $<10^9$/L	1.23 [0.8–1.6]	0.58 [0.48–0.70]	0.007
Apoptosis of lymphocytes (%)	37.6 [28.8–43.6]	47.9 [28.0–57.8]	0.3

Table 4. Lymphocyte apoptosis and mortality risk in COVID-19

Apoptosis of lymphocytes (%)	Se	Sp	PPV	NPV	LR+	LR–	RR	p
Threshold 55%	50	86	0.37	0.08	3.8	0.5	4.2 [1.0–17.2]	0.03

Note: Se, test sensitivity; Sp, test specificity; PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio of a positive test result; LR-, likelihood ratio of a negative test result; RR, relative risk; *p*, level of statistical significance.

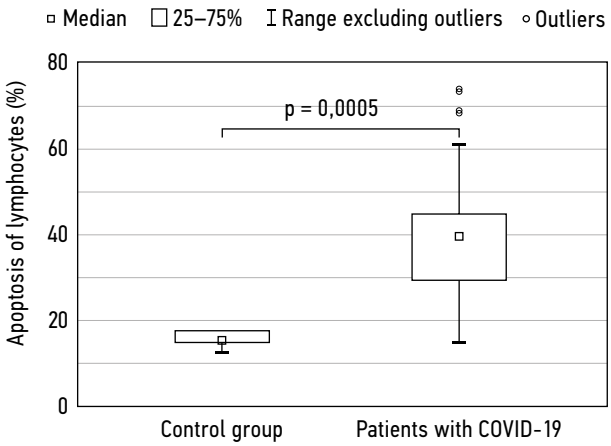

Fig. 1. Apoptosis of blood lymphocytes in patients with COVID-19 and in the control group

Virus-induced apoptosis, excessive pro-inflammatory cytokine synthesis, and lymphocyte sequestration in the lung tissue are the most possible causes of lymphopenia in COVID-19 [4, 7].

The hypothesis of virus-induced lymphopenia in COVID-19 is most appropriate considering the ability of SARS-CoV-2 to infect lymphocytes. Ren et al. showed that SARS-CoV-2 RNA is present in immune cells such as neutrophils, macrophages, and lymphocytes (T, B, and NK cells) [8].

However, SARS-CoV-2 RNA-positive immune cells did not express the angiotensin-converting enzyme type 2 (ACE2) receptor, which is a key protein for the virus to enter cells [9]. Some studies have reported extremely low ACE2 expression in lymphocytes [10]. Therefore, unlike the epithelial cells of the respiratory, gastrointestinal, and other systems, ACE2 receptors do not play a significant role in SARS-CoV-2 infection of lymphocytes.

The mechanisms of SARS-CoV-2 entry into lymphocytes is discussed. Early in the COVID-19 pandemic, several investigators demonstrated an alternative, ACE2-independent pathway of lymphocyte infection mediated by CD147 glycoprotein expressed on various cells, including T lymphocytes [11]. In 2022, Shen et al. found that the entry molecule for SARS-CoV-2 to infect lymphocytes was the leukocyte function-associated antigen-1 protein, which is solely expressed in leukocytes, including lymphocytes [12].

However, despite the ability of SARS-CoV-2 to enter lymphocytes, no evidence of viral replication in lymphocytes was observed. Moreover, several studies have demonstrated SARS-CoV-2-induced lymphocyte apoptosis [3, 12, 13]. CD4 lymphocytes are most susceptible to apoptosis in COVID-19 [14]. The degree of T-lymphocyte apoptosis in COVID-19 was reported to correlate with disease severity [15].

In the current study, the median lymphocyte apoptosis rate in patients with COVID-19 was 2.6 times higher than that in the control group ($p < 0.001$) and was significantly higher in patients with severe disease ($p = 0.02$). Additionally, lymphocyte apoptosis >55% in patients with COVID-19 was associated with the risk of death, indicating the high prognostic value of this parameter.

Notably, cell apoptosis is activated by the extrinsic pathway (through the expression of Fas receptors of the cell membrane) and intrinsic pathway (by reducing the membrane potential of the mitochondria) [14]. The extrinsic apoptotic pathway is mediated by caspase-8 and the intrinsic pathway by caspase-9. In the final stage, caspase-3 plays a critical role and is activated by caspase-8 and caspase-9 [16]. Ren et al. showed that in COVID-19, cell apoptosis occurs through the extrinsic pathway by induction of caspase-8 activation by the SARS-CoV-2 ORF3a protein [17].

In COVID-19, the clinical significance of lymphocyte apoptosis is related to viral clearance, and the resulting

apoptosis-induced lymphopenia may lead to immunosuppression and disease progression. This is supported by the pathomorphology of patients who died of COVID-19, which showed lymphoid tissue depletion [18, 19].

Activation of lymphocyte apoptosis and lymphopenia in severe COVID-19 may be caused by a significant inflammatory response characterized by increased levels of several blood pro-inflammatory cytokines (i.e., tumor necrosis factor α, interleukin (IL-1, IL-6, and IL-8) and other acute-phase inflammatory proteins (i.e., C-reactive protein, ferritin, and fibrinogen) [6, 20].

The ability of tumor necrosis factor α to induce apoptosis of human T lymphocytes was demonstrated in an in vitro experiment in 2002 [21]. In addition, IL-1β and IL-6 promote proapoptotic Fas receptor activation [22]. These indicate that the cytokine storm is a major cause of SARS-CoV-2-induced lymphocyte apoptosis.

An inverse correlation between interleukin-6 concentration and absolute lymphocyte count has been shown in COVID-19 patients [23]. In real clinical practice, to examine the degree of inflammatory response, blood is tested for acute phase inflammatory proteins such as C-reactive protein and ferritin, the synthesis of which is known to be induced by IL-6 [24]. High levels of these inflammatory markers have been notably correlated with COVID-19 severity and poor prognosis [25].

A moderate direct correlation was found between degree of lymphocyte apoptosis and blood ferritin level in COVID-19 patients (Spearman coefficient $p = 0.39$, $p < 0.05$).

This indicates the importance of anti-inflammatory therapy as a possible way to suppress excessive lymphocyte apoptosis. Some studies have shown that tocilizumab is associated with increased blood lymphocyte counts [23, 26].

Lymphocytes and respiratory epithelial cells and endothelial cells are susceptible to apoptosis in COVID-19 [27]. Furthermore, tissues from patients who have died from COVID-19 showed signs of apoptosis and pyroptosis, a form of cell death that combines signs of apoptosis and inflammation [28]. These pathomorphologic changes demonstrate the key role of hyperinflammation in the induction of cell death and organ dysfunction in patients with COVID-19, particularly those with acute respiratory distress syndrome. Therefore, anti-inflammatory therapy is critical to stop COVID-19-associated cytokine storm and lymphopenia, which may improve the prognosis of the disease.

CONCLUSION

1. COVID-19 is characterized by increased apoptotic activity of peripheral blood lymphocytes.

2. The degree of lymphocyte apoptosis correlates with disease severity; the highest levels were observed in severe COVID-19 patients, and a cell apoptosis rate >55% was associated with death.

3. Anti-inflammatory therapy for moderate and severe COVID-19 is based on the correlation between the degree of lymphocyte apoptosis and blood ferritin levels.

ADDITIONAL INFORMATION

Authors' contribution. Kh.S.Kh. — conceptualization, formal analysis, writing — review and editing, supervision; S.V.B. — methodology, validation; V.A.A. — writing — review and editing; A.R.G. — investigation, writing — original draft; A.E.E. — investigation, writing —original draft.

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