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Original Article

TNF-α, iNOS Augmentation Due to Macrophages and Neutrophils Activity in Samples from Patients in Intensive Care Unit with COVID-19 Infection

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ABSTRACT

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Background and Aims: Cells and secreted molecules by the innate immune system are the essential factors in the pathogenesis and determining the severity of inflammation in COVID-19 patients. Severe inflammation results from increased activity of neutrophils, macrophages, and other cells with their products. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) increases the severity and pathogenesis of the disease caused by the virus. Phagocytes are armed with inducible nitric oxide synthase (iNOS), that upon stimulation by proinflammatory cytokines augment an immune response against pathogens.

Materials and Methods: Two groups of patients were included with COVID-19 infection from the intensive care unit (ICU, n=52) and (non-ICU-care, n=54). Blood samples were collected to measure cells and serum parameters, including lymphocytes, neutrophils, platelet counts, accompanied with C-reactive protein, lactate dehydrogenase, TNF-α and iNOS levels.

Results: In the ICU group, increased white blood cells (p=0.048), decreased lymphocytes (p=0.0007), increased neutrophils (p=0.001), decreased platelets, increase serum levels for lactate dehydrogenase (p =0.0001), c-reactive protein (p=0.003), TNF-α (p=0.018), and iNOS (p=0.008) were statistically obtained. Positive correlations were calculated between TNF-α and iNOS (r=0.65, p=0.0002) and with c-reactive protein (r=0.52, p=0.003) and with lactate dehydrogenase (r=0.68, p=0.0001).

Conclusion: Inflammation due to macrophages and neutrophils activity in COVID-19 patients and increased mediators correlate with disease progression. It seems that control of the cell activity and their inflammatory cytokines would be considered for therapeutic goals. Changing the polarization of inflammatory macrophages to anti-inflammatory macrophages with therapeutic applications could prevent the severity of the provocative course of the disease.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus is the causative agent of coronavirus disease-19 (COVID-19) respiratory disease. It is a viral disease with various clinical symptoms, from asymptomatic to clinical signs similar to the common cold with fever and cough [1, 2]. However, a significant proportion of clinical symptoms are severe complications that require intensive hospital care and mechanical ventilation [3]. One of the most important causes of such a wide-ranging clinical condition is the increased response of the immune cells to the virus, the most important of which is inflammation and inflammatory mediators. Cells and secreted molecules by the innate immune system are among the essential factors in the pathogenesis and determining the severity of inflammation [4]. Cytokine storm is a phenomenon in patients with COVID-19 that reflects and results in severe inflammation due to over activation of immune cells such as macrophages and neutrophils as well as their products [1, 4]. Evidence from studies indicates the widespread presence of inflammatory cells such as neutrophils at the onset of the disease, associated with acute inflammation and its progression [2]. In addition, chronic inflammation with other inflammatory cells, such as M1 macrophages, is predominant in the affected tissue, such as the lungs [5, 6]. The effects of inflammation, such as cytokine storm, cause tissue destruction and organ failure. Moreover, in the peripheral blood of these patients, increased activity of innate immune inflammatory cells has been shown by considering white blood cells (WBCs). For instance, proinflammatory immunophenotype could be seen in blood WBC smears. In addition, their number is related to the severity of the disease [5]. The essential inflammatory mediators in the cytokine storm phenomenon are interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α) and so on [7]. The inflammatory cytokine TNF-α secreted by macrophages and with less intensity by other immune cells [8]. TNF-α is involved in the pathogenesis and tissue damage caused by the virus. In addition, the more increasing of this cytokine associate with exacerbates the severity of the disease [9]. Due to its significant role in cytokine storm and its presence in acute and chronic inflammation as well as tissue failure, it would be thought that TNF-α would be an option for being neutralized in the treatment process [8, 10]. In COVID-19 disease, an increase in this proinflammatory cytokine is associated with an increase in neutrophils and monocytes population in peripheral blood [11].

Inducible nitric oxide synthase (iNOS) is an enzyme synthesized by many cell types in response to inflammatory cytokines, mainly indicating macrophage and neutrophil activity. This enzyme is the primary catalyst of active nitrogen species and participates in the microbial defense process by producing nitrit oxide [12]. The action of this enzyme and its products from inflammatory macrophages of the lung could accelerate the thrombosis process [13]. Septic shock in mouse models has also been positively

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associated with increased levels of this enzyme [14]. iNOS plays an essential role in forming atherosclerotic plaques in the vessels of lung and heart tissues [15]. In this regard, another blood factor that shows the destructive effects of inflammation is the enzyme lactate dehydrogenase (LDH). In some infectious agents, especially viral infections, tissue damage is due to increased immune responses, followed by an increase in LDH [16]. Acute phase C-reactive protein (CRP) is another inflammatory factor, which is directly related to the extent and severity of inflammation in COVID-19 disease. The CRP increased in the blood in the early hours after infection and inflammation [17]. Accordingly, the significant threshold for serum CRP levels in viral infections such as COVID-19 for prognosis differs from other types of infections [18]. The main source of CRP secretion is tissue inflammatory macrophages (M1). And it has a positive association with an increase of inflammatory cytokines such as IL-6 and Granulocyte-macrophage colony-stimulating factor. Subsequently, the increased levels of CRP and cytokines cause more differentiation of monocytes and antigen-processing dendritic cells to become inflammatory macrophages [19]. This study aimed to evaluate the activity products of cells participating in inflammation, especially macrophages and neutrophils. For considering the importance of inflammatory cells in the pathogenesis of COVID-19 disease, based on measurable factors in the blood such as TNF-α, iNOS, CRP, LDH, and WBCs as indicators of enhancing the progression of COVID-19 antiviral immune responses in two groups of patients admitted to the intensive care unit (ICU) care wards and non-ICU care wards in University hospital.

Materials and Methods

This cross-sectional study was performed on patients with COVID-19 referred to Hospital (Shohadaye-Khalije-Fars) with the approval of the University ethics committee (IR.BPUMS.REC.1399.068), with received consent form. 106 patients were admitted with the definite disease after confirmation with real-time polymerase chain reaction (PCR) (viral nucleic acid extraction kit, South Korea, COVID-19 one step real-time PCR kit, Iran) was selected in April-May 2021. Patients were divided into two groups: those in the ICU-care and non-ICU. The patient blood samples were obtained, and the following laboratory tests were performed; CRP, LDH, TNF-α, iNOS in serums, and complete blood count (CBC) in their whole blood. Serum levels of TNF-α and iNOS were assayed by enzyme-linked immunosorbent assay (ELISA) for 43 patient samples with using (HumanTNF-α DuoSet, R&D, USA) and (human iNOS, CSB-E08148H, cusbio, USA). Serum CRP level was measured using a quantitative method (Biorex Fars, Iran) by autoanalyzer (Biotecnica BT3000). LDH measurement (Biorex Fars, BxC0420) using a quantitative method by autoanalyzer (Biotecnica BT3000), and CBC performed by hematology device (Sysmex, KX21N). For interpretation, the blood parameters, lymphocyte and neutrophil percentages, calculated and converted to absolute number for statistical use. All white blood cells

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(WBCs) except the lymphocyte population come to account for the name of neutrophils for easy interpretation. The amount of WBCs is based on the absolute number per microliter ($x10^3$ µl).

Statistical analysis

The SPSS statistics software was used to perform analysis of the collected data. An independent t-test was used to compare the mean between the groups. Spearman rank correlation coefficient test was used to compare correlation. Significant differences were considered for the tests with a P-value <0.05.

Results

We chose 106 patients admitted to the hospital in a group of 40-60 years old, (54 patients in the ward), and 52 patients in the ICU participated in this study. The difference for the ages was with P-value=0.18 between the groups. The population of both groups was included 25 men and 29 women in the non-ICU group and 28 males and 24 females in the ICU group. There was no difference between the groups in terms of gender and age. The data has been presented in table 1, showing mean ± SD after analysis.

There was a significant increase in WBCs in the ICU group compared to the non-ICU group (p=0.048). Lymphocyte percentages decreased in the ICU group (p=0.0007). Increased neutrophil count in the ICU group (p=0.04) was observed and a trend in decrease lymphocytes in the ICU group (p=0.1), but it does not approve statistically. Platelet counts were less in the ICU group but statistically did not reach a significant level (p=0.62). In addition, comparing the ratio of neutrophils to lymphocytes count (N/L ratio) shows the high ratio in the ICU group (p=0.005). Independent analysis was performed to compare the parameters between men and women in each group, but no statistical difference was detected (the data are not shown). There was a significant increase in the blood LDH in the ICU group (p=0.0001) and also CRP was augmented in the ICU group (p=0.003).

Table 1. The distribution of blood clinical laboratory factors in COVID-19 patients from ICU-care and non-ICU care in the University hospital in April-May 2021

	Non-ICU(n=54)	ICU-care (n=52)	p-value
Age (year)	49.4±6.7	51.82±7.9	0.18
WBC $x10^3$/µL	7.36±3.6	9.14±4.3	0.048
Lymphocyte percentage	21.51±10	13.33±8.8	0.0007
Lymphocyte $x10^3$/µL	1.38±0.74	1.08±0.73	0.1
Neutrophil $x10^3$/µL	5.94±3.4	7.84±4.2	0.04
Platelet×10^3/µL	268.8±169.7	243.8±81.5	0.62
Neutrophil/lymphocyte ratio	4.3±1.28	7.25± 2.3	0.005
TNF-α (Pg/mL)	17.03±18.5	33.26±24.6	0.018
iNOS (IU/ml)	4.373± 2.69	6.87± 3.17	0.008
CRP (mg/l)	35.96± 21.4	72.2± 56.9	0.003
LDH (IU/L)	365.6±110.3	639.5±275.7	0.0001

The data are presented as mean±SD
WBC= White blood count; TNF-α= Tumor necrosis factor -α; iNOS= Inducible nitric oxide synthase; CRP= C-reactive protein; LDH= Lactate dehydrogenase

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Serum levels of TNF-α in the ICU group were almost twice (p=0.018). For the iNOS serum levels, a significant increase was seen in the ICU group (p=0.008). A positive correlation was obtained between TNF-α and iNOS with (r=0.65, p=0.0002). Also, for CRP and TNF-α with (r=0.52, p=0.003) and for LDH with (r=0.68, p=0.0001) respectively.

Discussion

In this study, the levels of inflammatory cytokines TNF-α and iNOS significantly augmented in ICU COVID-19 patients compared to non-ICU patients, which indicates the increase and progression of inflammation and disease. Various studies have reported increased levels of TNF-α for COVID-19 patients [20]. It is considered an aggravating factor in the pathogenesis of the disease. Drugs that inhibit this cytokine or its receptor, also known as an anti-inflammatory, have been used for the treatment. The Stebbing et al. study shows that both antiviral and anti-TNF-α drugs have a better effect on the recovery of COVID-19 patients [21]. In addition, anti-TNF-α drug therapy in patients with rheumatoid arthritis has shown to be effective in treating COVID-19 [10]. Also, the main cellular source in the production and secretion of these inflammatory mediators is macrophages and, to a lesser extent, neutrophils [22]. The inflammation can probably be managed by controlling the activity of these cells [11, 23]. It has been reported to increase the ratio of neutrophils to lymphocytes in the peripheral blood of these patients, which reflects the high activity of TNF-α producing cells [5, 24]. As we show that the increased TNF-α was associated with increasing the ratio of neutrophils to lymphocytes in patients admitted to the ICU than in patients admitted to the ward. In these patients, the level of iNOS in the intensive care group is significantly higher than the non-ICU group. The level of iNOS was also positively associated with clinical symptoms and disease severity. It indicates an increase in the activity of inflammatory macrophages in these patients. Studies on COVID-19 have shown that iNOS increases the expression of angiotensin-converting enzyme 2, the COVID-19 virus receptor on cells, and intensifies chronic inflammatory condition by macrophages [12]. In addition, iNOS is produced from other cellular sources, such as neutrophils, and exacerbates inflammatory conditions [25]. In this study, by examining the WBC parameters of COVID-19 patients, an increase in myeloid cells was observed and an increase in their ratio to lymphocytes, which interprets the consequence of increased iNOS production. The findings of Chen et al. also indicate an increase in iNOS protein in the lungs of COVID-19 patients. The rate of iNOS expression has been very high in people who have died of COVID-19 [26]. Increased inflammatory cytokines and iNOS are direct mediators of inflammation progression due to increased activity of immune cells such as macrophages [14]. Elevated LDH has been reported for severity and mortality by Henry et al. (performing a pooled analysis) with a cut-

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off point above 253U/L [16]. Higher levels were associated with the severity of COVID-19 disease and its mortality [27]. Therefore, serum LDH level can be considered a predictor of the involvement of several major organs following viral infection and activation of the immune cells [16]. The most important tissues involved in rising LDH are heart, lung, and kidney tissue. Acute phase protein CRP is reflecting acute inflammatory conditions in the viral infection. Increases of this protein indicate an association of CRP levels with disease severity [17]. Also, people who died of the virus had ten times more CRP than those who had just recovered from the disease [28]. Significant range in COVID-19 disease is on the critical condition of CRP≥40 mg/L as a deciding factor in the prognosis of infection progression [17]. Tan et al. also showed that both CRP and LDH factors increased in patients with COVID-19 before changes in computed tomography scan results [28]. Accordingly, these two indicators including CRP and LDH at the time of patient admission play a decisive role in predicting the course of the disease and considering appropriate treatment. Poggiali et al. also showed that increasing CRP and LDH above their cut-off rate is strongly associated with decreasing the PaO2/FiO2 ratio [29]. The present study also showed that CRP level in the ICU group was higher than the non-ICU patients (CRP≥40 mg/L). Macrophage inflammatory product, such as IL-6 has also reported as a valid biomarker in the prognosis and estimation of mortality in COVID-19 disease [30]. But CRP is important due to the availability of its faster assay and examination of the patient's condition. Therefore, it can be concluded that having several biomarkers which predict the severity of the disease would be vital in monitoring the patients. Xie et al.'s study shows that people with low oxygen saturation (less than 90%) had an increased level of CRP (76 mg/L) compared to people with adequate oxygen levels [31].

The phenomenon of platelet dysfunction has been reported in patients with COVID-19, and for this reason, platelet count and their function have been evaluated in many studies of COVID-19 related disease [32]. In our research, we also saw a decrease in platelet count in people in the intensive care unit. Moreover, in inflammatory conditions, tissue factor expression increases, and platelet activation occurs. Platelets affect the cells of monocytes, forcing them to express tissue surface factor and cause the coagulation process [33]. So, it can be concluded that the establishment of chronic inflammation due to macrophages and less important neutrophils in these patients can be adjusted by examining and analyzing the inflammatory markers mentioned here in favor of control the disease.

One probable strategy can be a polarization of inflammatory macrophages (M1) to alternative macrophages (M2), perhaps with the goal of eliminating the complications of tissue failure and death at the very beginning of the disease.

For this purpose, some studies have targeted macrophage polarization. For instance, using melatonin as a proposed treatment for altering

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the metabolism of inflammatory macrophages and convert them to macrophages with anti-inflammatory properties [34]. It has also been used to polarize macrophages using low-dose radiation [35]. Moreover, to neutralize the population of inflammatory macrophages by using autologous M2 autologous anti-inflammatory macrophage population [36]. It would be possible that the patients in the early stages of the disease progression can probably be prevented from the severity of the process and the spread of inflammation.

Conclusion

It could be concluded that inflammatory factors such as TNF-α and iNOS along with CRP and LDH parameters can be predictors of the severity of inflammation during COVID-19 disease progression. Also, by regulating the function of innate immune cells as a primary cellular source of producing the inflammatory factors, it could be beneficial in controlling these cells by using autologous M2 macrophage population. It would be possible that the patients in the early stages of the disease progression can probably be prevented from the severity of the process and cytokine storm.

Conflict of Interest

The authers declare no conflict of interest.

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