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Oxidative stress, DNA damage, and inflammation in COVID-19 patients

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ABSTRACT

OBJECTIVE: Severe inflammation and oxidative stress seen in COVID-19 patients cause cumulative antiviral effects, and serious inflammation increases tissue, oxidative damage, and DNA damage. Therefore, in this study, oxidative stress, DNA damage, and inflammation biomarkers were investigated in patients diagnosed with COVID-19.

METHODS: In this study, blood samples were obtained from 150 Covid-19 patients diagnosed by polymerase chain reaction and 150 healthy volunteers with the same demographic characteristics. Total oxidant status (TOS), total antioxidant status (TAS), total thiol (TT), native thiol, and myeloperoxidase (MPO) activities were measured by photometric methods. The levels of tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6), which are inflammation markers, were measured by the ELISA method using commercial kits. The genotoxic effect was evaluated by Comet Assay.

RESULTS: The oxidative stress biomarkers; Disulfide, TOS, MPO, oxidative stress index, and IL-1β, IL-6, and TNF-α levels of inflammation biomarkers and the DNA damage in COVID-19 patients were increased ($p<0.001$), and the levels of TAS, TT, and NT In COVID-19 patients were decreased ($p<0.001$).

CONCLUSION: In COVID-19 patients, induced DNA damage, inflammation, and oxidative stress can guide the prognosis and treatment strategies of the disease.

Keywords: COVID-19; genotoxicity; oxidative stress.

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Coronaviruses (CoV) are single-stranded, positive polarity enveloped RNA viruses that cause a variety of illnesses as middle eastern respiratory syndrome (MERS-CoV) and severe acute respiratory syndrome (SARS-CoV). New mutated version of the virus has been accepted as SARS-CoV-2. The virus is in the Sarbecovirus subgenus within the beta coronavirus genus, including SARS-CoV and MERS-CoV, and was named COVID-19 by the World Health Organization [1]. The mechanism of infection is that the virus binds to Angiotensin-Converting Enzyme 2 (ACE2). This complex is taken up and replicated by the host cell [2]. The onset of symptoms (incubation period) from exposure to COVID-19 is 2–14 days. Many people with confirmed COVID-19 infection cause severe acute respiratory disease with fever, cough, and short-

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ness of breath [3]. The virus is mortal in the elderly and people with chronic diseases namely hypertension, cardiovascular disease, and diabetes, with a mortality rate of about 3%. Severe cases are hospitalized and receive "supportive treatment." The organ in which the COVID-19 virus is most effective is the lungs. The virus travels through the airways to the lungs, binds to the receptors of ACE2 on the cell surface of the alveoli. After this, the virus enters the cell, multiplies inside, and damages the cells [2, 4]. Acute inflammation that caused by the virus is a severe syndrome of acute respiratory failure syndrome, which can be fatal.

Although it has been a year since the virus appeared in the World, the long-term effects of the virus are still a matter of curiosity. Severe inflammation and oxidative stress seen in patients cumulatively cause an antiviral effect, as well as an extreme increase in tissue inflammation, oxidative damage, and DNA damage [5, 6]. In this study, oxidative stress, DNA damage, and inflammation biomarkers were investigated in patients diagnosed with COVID-19.

Highlight key points

- Severe inflammation and oxidative stress seen in patients cumulatively cause an antiviral effect, as well as an extreme increase in tissue inflammation, oxidative damage, and DNA damage.
- Total thiol and native thiol values were statistically significantly lower in COVID-19 patients, and disulfide values were significantly higher.
- While oxidative stress and inflammation are induced in COVID-19 patients, antioxidant defenses decrease.
- Increased inflammatory markers IL1β, IL6, and TNFα are associated with COVID-19 disease severity.

MATERIALS AND METHODS

The Study Design

The study was performed between April and June. One hundred and fifty patients (120 men + 30 women) who were applied and diagnosed by polymerase chain reaction for COVID-19 at the Emergency Medicine Department of X. After 150 patients signed, the informed consent form and routinely requested blood samples were studied, the remaining inert blood was investigated. The healthy people with the same demographic characteristics without any chronic diseases were included as the control group. Our article has been prepared in conformity with the Declaration of Helsinki. The ethics were approved by University of Health Sciences Turkiye, Hamidiye Scientific Research Ethics Committee with decision number 21–267.

Blood Sample Collection

Approximately 3 mL of blood from the patients was taken into sterile blood tubes with EDTA. A volume of 200–400 μL was drawn and transferred to 1.5 mL centrifuge tubes from this blood. The residual EDTA blood was 10 min centrifuged at 3000xg. After centrifugation, its plasma was stored at -80°C until analysis.

Alkaline Single Cell Gel Electrophoresis

Leukocyte DNA damage was analyzed by alkaline single-cell gel electrophoresis, namely, the Comet Assay method [7]. For this purpose, 6 μL of whole blood from the thawed whole blood was mixed with low melting temperature agarose (0.7%), then embedded on slides covered with agarose gel (1%) with a normal melting temperature. The coverslip covered and permitted to solidify in a cold environment. After the gel solidified, the coverslips were removed from the slide, and cells were lysed in a lysis buffer for at least 4 h. Subsequently, they were electrophoresed (300 mA) for 20 min in an alkaline buffer (pH 13). The cells stained with Ethidium Bromide (5 mg/mL) were examined by fluorescence microscopy (Excitation DB: 546 nm, Emission DB: 20 nm) after electrophoresis. Tail density (tail %) in DNA was analyzed as a DNA damage signal. Comet analyzes were performed using Comet Assay analysis program IV (Perceptive Instruments, Suffolk, UK), counting an average of 50 cells.

Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) Measurement

The total antioxidant levels of the plasma samples were studied using the method developed by Erel [8]. The test is based on the degradation of the blue-green color formed by ABTS (2,2'-azinobis-3-ethylebenzothiazoline-6-sulfonate) radical with antioxidants in the sample. ABTS is incubated with a peroxidase containing myoglobin (HX-Fe+) and H_2O_2 to form the ABTS+ radical. The resultant ferryl myoglobin reacts with ABTS to form the ABTS+ radical, it is blue-green in color. This formed color is inhibited according to the ratio of antioxidants in a sample and measured with a Varioskan Multimode

Reader (Thermo Scientific, USA) at 600 nm. Ascorbic acid was used as a standard in the calculation of TAS.

The plasma total oxidant levels were determined using the method developed by Erel [9]. The total oxidant test is depending on the oxidation of ferric ion to ferrous ion in the availability of diverse oxidant species. To make it ironic, the oxidants oxidize the iron-a-dianisidine complex. The produced ferric ion makes a colored complex of xylenol orange-ferric ion. In a sample, the amount of oxidant will be associated with the intensity of color. This color-changing was measured with a Varioskan Multimode Reader at a wavelength of 530 nm. As a standard for TOS calculation, H_2O_2 was used. The oxidative stress index (OSI) was calculated as TOS/TAS.

Thiol-disulfide (DIS) Homeostasis

The "Modified Ellman method" of Erel and Neselioglu was used for total and free thiol measurement [10]. For total thiol (TT) measurement, 10 µL of R_1 (reagent 1), and for free thiol measurement, 10 µL of R1' was added to 10 µL sample. Then, by adding R_2 and R_3, the first absorbance (A1) reading was done spectrophotometrically at 415 nm wavelength a Multimode Reader. The second absorbance (A2) reading occurred at the same wavelength in the 10th min when the reaction plateaued. The measurement was completed by obtaining the A2-A1 absorbance difference. In calculating total and free thiol levels, 5-thio-2-nitrobenzoic acid, 14.100 mol/L^{-1}cm^{-1} was used. The DIS level was calculated as µmol/L using the below formula:

Myeloperoxidase (MPO) Enzyme Activity Measurement

The plasma MPO enzyme activity of the samples was determined by the modified o-dianisidin-H_2O_2 method for 96-well plates. Plasma samples (20 µL) were added to 0.53 mmol/L o-dianisidin dihydrochloride and 50 mmol/L of potassium phosphate buffer with pH 6.0. Then incubated for 10 min at room temperature. After incubation, the change in absorbance (ε= 10062/M/cm) was measured. Results are expressed in U/L for 10 min.

Inflammation Markers

The levels of tumor necrosis factor-alpha (TNF-α) (BT Laboratory E0082Hu), IL1β (BT Laboratory E0143Hu), and IL6 (BT Laboratory E0090Hu) were measured by commercially purchased ELISA kits by the photometric method.

Statistical Analysis

Parametric data were expressed as mean±standard deviation, while non-parametric data as the interquartile range (IQR). Shapiro–Wilk test was used for normality distribution. The difference between the two parameters was calculated using the Mann–Whitney U test. To compare more than two independent parameters, Kruskal–Wallis test was used. Using the Spearman rank correlation coefficient, the correlation between two variables was evaluated. The chi-square test was used to evaluate the categorical data. Moreover, the $p<0.05$ was regarded as statistically significant. All statistical analyses were performed by the SPSS version 25.0 program (IBM, Armonk, NY, USA).

RESULTS

Considering the demographic characteristics of the study groups, no significant difference was observed between the patient and control group in terms of gender (20W;130M) and mean age (35.60±10.10; 34.31±9.35).

Oxidative Stress Biomarkers

The DIS, TOS, MPO, and OSI in which oxidative stress was indicated oxidative damage were significantly increased in the COVID-19 patients than the healthy control group ($p<0.001$). TAS, TT, and NT levels, which indicate antioxidant capacity, were statistically significantly decreased in COVID-19 patients ($p<0.001$), see Table 1.

Inflammation Biomarkers

The levels of inflammation biomarkers TNF-α, interleukin 1 beta, and interleukin 6 (IL-6) were shown in COVID-19 patients. As seen in Table 2, the data increased statistically significantly compared to the healthy control group ($p<0.001$).

Comet Assay

To evaluate DNA damage in patients diagnosed with COVID-19, comet assay method was used. The amount of damage is given as % tail density. The mean tail percentage for COVID-19 patients was 43.10±10.17, and for the control group was 4.04±0.82. In COVID-19 patients, DNA damage increased statistically according to the healthy control group ($p<0.001$), Figure 1.

Table 1. Oxidative stress biomarkers in COVID-19 patients and healthy controls

	Oxidative stress biomarkers		p
	Healthy control, Mean±SD	COVID-19, Mean±SD	
TOS μM H_2O_2 Eq./L	9.61±2.94	15.10±2.76	0.001
TAS μM Ascorbic acid Eq./L	1.12±0.19	0.73±0.15	0.001
OSI AU	9.14±3.96	22.16±8.89	0.001
MPO U/L	54.56±16.01	140.63±21.70	0.001
TT μmol/L	560.43±60.07	475.50±63.39	0.001
NT μmol/L	457.52±84.97	312.07±62.05	0.001
DIS μmol/L	51.45±33.70	81.72±28.19	0.001

SD: Standard deviation; TOS: Total oxidant status; TAS: Total antioxidant status; OSI: Oxidative stress index; MPO: Myeloperoxidase; TT: Total thiol; NT: Native thiol; DIS: Disulfide.

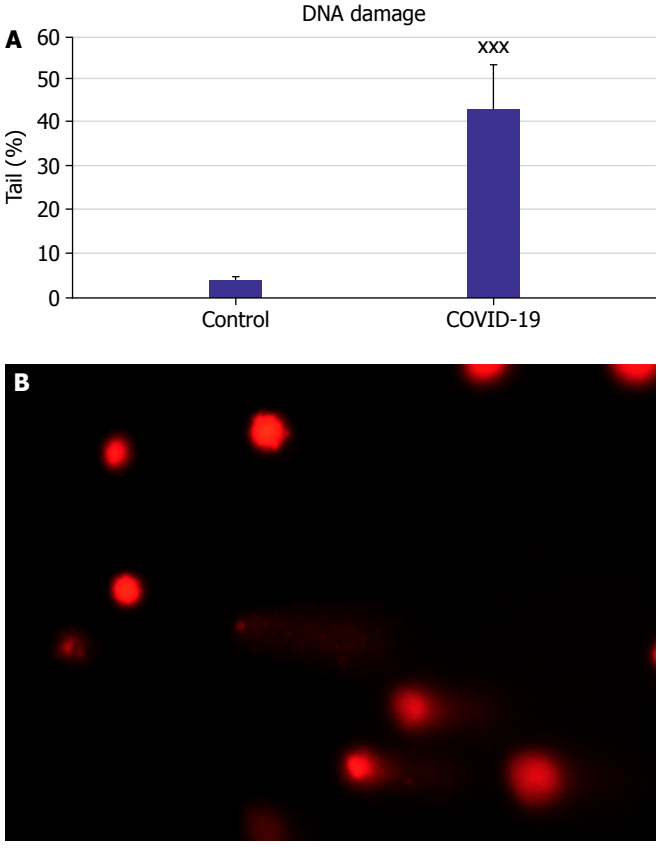

Figure 1. Effect of COVID-19 patients on DNA damage. **(A)** Percentage DNA in tail determines in COVID-19 patients and control group. **(B)** Representative image of DNA damage pattern in COVID-19 patient number 34. Statistically significant differences of relative values in COVID-19 patients $^{x}p<0.05$, $^{xx}p<0.01$, and $^{xxx}p<0.001$ was compared to control. Data are indicated as the mean±standard deviation.

Table 2. Inflammatory biomarkers in COVID-19 patients and healthy controls

	Inflammatory biomarkers		p
	Healthy control Mean±SD	COVID-19 Mean±SD	
IL-1β pg/L	103.35±18.24	363.30±124.36	0.001
IL-6 ng/L	64.85±36.25	326.08±84.47	0.001
TNF-α ng/L	81.70±37.04	219.43±49.90	0.001

SD: Standard deviation; IL-1β: Interleukin 1βeta; IL-6: Interleukin 6, TNF-α: Tumor necrosis factor α.

DISCUSSION

In this study, oxidative stress, DNA damage, and inflammation biomarkers were investigated in patients diagnosed with COVID-19. Oxidative stress occurs due to disturbances in the stabilize of production and destruction of ROS. It is characterized by overcoming and damaging the repair capacities of cells through the oxidation of DNA, membrane lipids, and structural proteins [11]. High ROS levels due to respiratory viral infections are related with cytokine production, cellular damage, and oxidative stress or redox imbalance. Virus infection produces large amounts of free radicals, and high ROS levels with these depletion antioxidant mechanisms are crucial for virus replication [12, 13]. Current studies state that oxidative stress is a crucial factor increasing the severity of COVID-19 in patients with lung dysfunction and the

cytokine storm from SARS-CoV-2 infection [5, 14, 15]. According to obtained data of our study, OSI levels were significantly increased in COVID-19 patients. These results were confirmed with the literature.

MPO is a crucial enzyme secreted mainly by activated neutrophils and is characterized by pro-inflammatory attributes. The property of COVID-19 is that infiltrating neutrophils release MPO, which activates several pathways leading to cytokine regulation and the production of ROS [16]. A study by Guéant et al. [17] observed elevated MPO-DNA blood levels in positive patients, indicating that this is a sensitive marker of the early stage of COVID-19. Our results demonstrated that the MPO levels increased in COVID-19. These results are consistent with the literature.

Thiols undergo oxidation reactions through oxidizing agents and forms DIS bonds, which can be reduced back to thiol groups, thereby maintaining thiol-DIS homeostasis [18, 19]. In this homeostasis, thiol plays a crucial role in the protection of antioxidants, protein synthesis, cellular growth and proliferation, apoptosis, and cellular signaling mechanism [20]. The previous studies have demonstrated that viral glycoprotein entry is affected by the thiol-DIS balance on the cell surface [21, 22]. According to the data, we obtained in our study, TT and NT values were statistically significantly lower in COVID-19 patients, while DIS values were significantly higher.

Cytokine storm is the extensive and uncontrolled release of pro-inflammatory cytokines and usually occurs as systemic inflammation and multi-organ failure [23]. The levels of IL-2, IL-7, IL-10, TNF-α, and MCP-1 were observed to be higher in severe COVID-19 patients followed in the intensive care unit compared to other patients [5, 24, 25]. In a study by Chen et al. [26], they characterized and compared the immunological features of COVID-19 cases with several disease severity. As a result, they reported that IL-6, IL-10, and TNF-α levels were increased when the serum levels of patients with a severe disease course were compared with moderate severity patients. In our results, IL1β, IL6, and TNFα levels of inflammation biomarkers were statistically significantly increased in COVID-19 patients. In light of all these findings, increased inflammatory markers are related to COVID-19 disease severity.

The comet assay is a method to evaluate DNA damage. This study demonstrated that it induces DNA damage in COVID-19 patients and observed that it is more susceptible to damage than healthy controls. Studies are showing that increased intracellular ROS in viral infection triggers DNA damage [27]. In a study by Singh et al. [28], mitochondrial disruption in SARS-CoV-2 infected lung cell lines were proven to increase inflammation and severity in COVID-19-related sepsis. The only limitation of our study was that the relationship between the radiological prevalence of errors and biomarkers could not be examined.

Conclusion

Our study concluded that while antioxidant defenses decrease in COVID-19 patients, oxidative stress and inflammation are induced. Therefore, induced oxidative stress, inflammation, and DNA damage in COVID-19 patients can guide the prognosis and treatment strategies of the disease. The effects of this increased oxidative stress and inflammation-induced DNA damage in patients diagnosed with COVID-19 should be followed for a long time.

Ethics Committee Approval: The University of Health Sciences Turkiye, Hamidiye Faculty of Medicine Scientific Research Research Ethics Committee granted approval for this study (date: 02.04.2021, number: 21–267).

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Authorship Contributions: Concept – GB; Design – GB, SC; Supervision – GB, EMG; Fundings – EMG; Materials – EMG, SA; Data collection and/or processing – EMG, SA; Analysis and/or interpretation – EMG, SA, KB; Literature review – GB, SC; Writing – EMG, KB; Critical review – GB, SC, EMG.

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