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Oxidative Stress Mediates Cross-Talk between the Thioredoxin System and NLRP3 Inflammasome in Patients with COVID-19

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Background: COVID-19 is known as a fatal respiratory disease with an inflammatory basis. Imbalance of the thioredoxin system in peripheral blood mononuclear cells (PBMCs) may cause NLRP3 inflammasome activation and interleukin-1β production.

Materials and Methods: Forty patients with COVID-19 and forty healthy individuals were included in this case-control study. All participants had previously received two doses of the coronavirus vaccine. PBMCs were isolated, and the expression of thioredoxin-interacting protein (TXNIP), thioredoxin-1 (TRX-1), NLRP3, caspase-1, and IL-1β genes was determined. Protein levels of TXNIP, NLRP3, and IL-1β were measured. Moreover, to confirm the involvement of oxidative stress in evoking inflammation in COVID-19, PBMCs were isolated from three independent healthy subjects, cultured *in vitro*, and infected with the SARS-CoV-2 virus in the presence or absence of vitamin C. Reactive oxygen species (ROS) and IL-1β levels were determined in cultured PBMCs.

Results: Oxidative stress was higher in COVID-19 patients. The mRNA expression and protein levels of TXNIP, NLRP3, and IL-1β were significantly higher in PBMCs of COVID-19 patients compared with the control group, whereas the expression of TRX-1 was found to be lower in patients. The enhancement in ROS, TXNIP protein, and NLRP3 gene expression levels was positively associated with increased IL-1β.

Conclusion: The concurrent elevation of ROS, TXNIP, NLRP3, and IL-1β suggests the potential role of the thioredoxin system in activating the NLRP3 inflammasome and generating inflammatory IL-1β in patients with COVID-19. In addition, vitamin C, as an adjunct therapeutic agent, beneficially suppressed oxidative stress and inflammation in infected PBMCs.

Keywords: COVID-19; Inflammasomes; Interleukin-1beta; Oxidative stress; Thioredoxins

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious coronavirus that first appeared in late 2019 and triggered the pandemic of acute respiratory disease known as "coronavirus disease 2019 (COVID-19)", which continues to endanger human health and safety, so that as of Feb 26, 2024, there had been more than 6.9 million fatalities and over 769 million verified cases globally (1-3).

The high viral load of the virus during the viremia phase and its high potency in recognizing and binding to angiotensin-converting enzyme 2 (ACE2) receptor lead to the high transmissibility of COVID-19, and result in the infection of human alveolar epithelial cells (3-5). Breathlessness and mild cough to severe acute respiratory distress syndrome are all consequences of respiratory system impairments. The first period of viremia is followed by acute organ injury. COVID-19 induces pneumonia and Acute Respiratory Distress Syndrome (ARDS) by disrupting the respiratory system and relying on the ACE2 binding sites in the acute phase (6). Additionally, it is believed that in viral infections, oxidative stress, which increases reactive oxygen species (ROS), is a significant cause of ARDS (7,8).

Oxidation of free thiol groups to disulfides, under the mechanism of oxidative stress, seems to increase the affinity of surface protein spike (S; spike) in SARS-CoV and SARS-CoV-2 for the ACE2 receptor and, as a result, increases the intensity of infection (9, 10). Reactive oxygen species play prominent biological roles in cell signaling pathways (redox signaling), thiol switches, and regulation of inflammatory cytokines and growth factors, as well as playing an essential role in the balance of factors involved in the oxidative stress system, such as TAC, TOS, and MDA (11). While there is ample proof of raised ROS and oxidative stress in a wide range of viral diseases, there is little evidence about the imbalance in biological systems involved in cellular oxide/redox status in SARS-CoV patients (12,13).

The increased oxidative stress that results in serious lung injury in severe acute respiratory syndrome is associated with immune system activation and the activation of inflammatory signaling pathways, such as the NF-kB pathway (14). COVID-19 infection causes an imbalance in the immune system due to the abundant release of inflammatory-promoting cytokines, including IL-1β, leading to a cytokine storm (15). Therefore, it can be postulated that oxidative stress and inflammation play crucial roles in the pathogenesis of SARS-CoV infection (7). An endogenous inhibitor of thioredoxin (TRX), also known as thioredoxin binding protein-2 (TRP-2), is key to cellular redox regulation and the homeostasis of the thioredoxin system (16). TXNIP inhibits the activity of TRX under normal physiological conditions by binding to it. When TXNIP is overexpressed in response to oxidative stress and dissociates from TRX, it activates redox-dependent signal pathways, including nucleotide-binding oligomerization domain receptors containing pyrin domain 3 (NLRP-3) inflammasomes (17, 18). The NLRP3 inflammasome is composed of three components: NLRP3 (the sensor molecule), CARD (ASC, the adaptor factor), and pro-caspase-1 (the effector protein) (19).

IL-1β is an inducible cytokine mainly secreted by monocytes, macrophages, and neutrophils. IL-1β remains intracellular when synthesized as pro-IL-1β because it lacks a signal peptide. Pro-IL-1β requires processing by caspase-1 to be converted to mature IL-1β. Oligomerization of NLRP3 by assembly of its components activates pro-caspase-1 to active caspase-1, which in turn initiates the processing and release of pro-inflammatory cytokine IL-1β (20).

Peripheral Blood Mononuclear Cells (PBMCs) are a suitable tissue for a variety of studies, such as immunological, molecular, and pharmacogenomic. PBMCs are permissive to SARS-CoV infection, allowing effective viral replication (19). Hence, the inflammation and oxidative stress in PBMCs may result in the activation of the oxidative stress system and, ultimately, the cytokine storm. Inflammation plays a significant role in COVID-19 disease, and TXNIP appears to activate NLRP3 inflammasome signaling in PBMCs, leading us to hypothesize that an imbalance in the thioredoxin system (TXNIP/TRX-1) increases inflammation in patients with COVID-19 disease, resulting in a cytokine storm by activating NLRP3 inflammasomes and releasing IL-1β. To confirm this hypothesis, it is investigated whether potent antioxidants such as vitamin C inhibit cellular oxidative stress and downregulate the NLRP3 pathway, leading to the suppression of IL-1β production in PBMCs infected

with SARS-CoV-2 in culture conditions. Thus, this research investigated the relationship between the thioredoxin system and the inflammatory complex in the pathogenesis of COVID-19 patients by measuring the mRNA expression of TRX, TXNIP, NLRP3, IL-1ß, and caspase-1 (as factors involved in inflammation), total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), and malondialdehyde (MDA) levels (as factors involved in oxidative stress) as well as determining TXNIP, NLRP3 and IL-1β protein levels in PBMCs isolated from COVID-19 patients and healthy control subjects. In addition, to confirm the role of oxidative stress in activation of NLRP3 inflammasome and pathogenesis of COVID-19, PBMCs were infected with the SARS-CoV-2 virus in culture conditions, and the beneficial therapeutic effects of Vit C in the infected cells were further investigated.

MATERIALS AND METHODS

Study population and study design

In the first phase of the study, a case-control investigation was carried out where forty COVID-19 patients with a positive PCR test were assigned as case individuals. In addition, 40 healthy participants with a negative PCR test (whose PCR examinations were required by international air flight policy during the COVID-19 pandemic) were considered control subjects. All participants had records of two-dose anti-COVID-19 vaccination. Participants were between 18 and 70 years old, and no significant difference was observed in the average age between patients (49.63 ± 8.7 years) and control subjects (50.02 ± 8.8 years). Blood samples were collected from all participants, and PBMCs were isolated. The study excluded individuals with diabetes, recent acute myocardial infarction, any type of malignancy, any recent major surgery, kidney or liver diseases, and those suffering from final-stage heart failure. All patient information, including demographics, anthropometrics, risk factors, medications, and clinical characteristics, was recorded. Written informed consent was obtained from all participants after explaining the study's nature.

In the second phase of the study, to confirm the involvement of oxidative stress in evoking cytokine release in COVID-19 patients, PBMCs were isolated from a set of three independent healthy subjects and infected with the SARS-CoV-2 virus in culture conditions. The infected cells were then treated with vitamin C as an antioxidant, and further analysis was carried out as described below.

The study received University Approval (UMSHA.140104072329) and was approved (IR.UMSHA.REC.1401.236) by Hamadan University of Medical Sciences Research Ethics Committee (Hamadan-Iran). All experiments carried out in this study were in agreement with the Declaration of Helsinki (version 2013) and with the ethical standards of the National Research Committee.

Sample collection

Ten milliliters of blood samples were collected in tubes containing EDTA anticoagulant from patients and control subjects (in the first phase of the study) and from independent healthy individuals (in the second phase of the study). PBMCs were isolated and used for molecular analysis, in vitro infecting of the cells with SARS-CoV-2, and further experiments using class-III virus laboratory facilities of the Pasteur Institute (Tehran, Iran).

Isolation of PBMCs

Venous blood (10 ml) was collected into EDTA-containing tubes (Merck, Germany). PBMCs were then isolated using Ficoll-Paque (Sigma-Aldrich, UK) and density gradient centrifugation. The ROS content of the cells, as well as TAC, TOS, and MDA levels, the mRNA expression levels of TRX-1, TXNIP, NLRP3, caspase-1, and IL-1β, and the protein levels of TXNIP, NLRP3, and IL-1β were determined in PBMCs.

Measurement of ROS level in PBMCs

Reactive oxygen species levels were determined using the Kiazist ROS Assay kit (Kiazist Life Sciences, Iran), according to the manufacturer's instructions. Briefly, PBMC cells were cultured in a 96-well U-bottom plate at 25,000 cells per well to reach the desired confluency (80-90%); then, the cells were washed with ROS buffer, and the

supernatant was discarded. Next, 100 µl of DCFDA (dichlorodihydrofluorescein diacetate) working reagent was added to each well, and cells were incubated at 37°C for 45 min in the dark. Then the solution was removed, ROS buffer was added to each well, and the fluorescence was immediately measured as emission/excitation at 535/485 nm. In addition, 100 µl of oxidized DCFDA working solution was dispensed into two wells as a control to obtain the high fluorescence level. Finally, the cells were analyzed based on the difference between the mean fluorescence intensity of ROS/$O^{2\bullet-}$ in PBMCs of patients and healthy subjects by FlowJo software (BD Biosciences, USA), and results were reported as mean fluorescence intensity (MFI).

Determination of total antioxidant capacity (TAC) in PBMCs

According to the manufacturer's instructions, the TAC assay kit (Kiazist Life Sciences, Iran) was used to determine antioxidant capacity in PBMC samples. An intense color, detectable at 450nm, is detected after ferric tripyridyltriazine is reduced to ferric tripyridyltriazine using the ferric-reducing antioxidant power (FRAP) method. Using the standard curve, the concentrations of the samples were expressed as nmol equivalents to Trolox per mg.

Determining the total oxidant status (TOS) in PBMCs

According to the instructions provided by the manufacturer, the Kiazist TOS kit (Kiazist Life Sciences, Iran) was used to determine the total oxidative status in PBMC samples. Molecular oxidants oxidized Fe^{+2} to Fe^{+3} and produced chromogens at 545 nm, allowing the detection of the chromogens. A nmol/mg value was finally assigned to the TOS levels.

Determination of lipid peroxidation in PBMCs

Levels of malondialdehyde (MDA), as the final product of lipid peroxidation, were measured in PBMC samples using a lipid peroxidation assay kit (Kiazist Life Sciences, Iran) according to the manufacturer's instructions. Using MDA and thiobarbituric acid as reactants, a chromophore complex was generated. A nmol/mg value was finally assigned to the MDA levels.

Measurement of gene expression levels, including TXNIP, TRX 1, NLRP3, Caspase-1, and IL-1β, using RT-qPCR

Total RNA was extracted from isolated PBMCs using RNX-Plus (Sinaclon, Iran). The quality and concentration of total RNA were determined using agarose gel electrophoresis and a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA), respectively. One microgram of the extracted RNAs was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Pars Tous kit, Iran) on a GeneAmp™ PCR System 9700 thermal cycler (Applied Biosystems Corporation, Massachusetts, USA) using random primers and oligo-(dT), and the resulting cDNA samples were stored at −20 °C.

The expression levels of TXNIP and TRX-1, components of the thioredoxin system, as well as NLRP3, caspase-1, and IL-1β, members of the inflammasome system, were assessed in PBMCs using RT-qPCR. AlleleID® v6.0 software (Premier Biosoft Corporation, USA) was used to design gene-specific primers, and online primer blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to confirm their specificity to the corresponding genes. For TXNIP, the primer set was 5′-CAGCCAACAGGTGAGAATGA-3′ (forward) and 5′-TTGAAGGATGTTCCCAGAGG-3′ (reverse). For TRX-1, the forward and reverse primer sequences were 5′-AGCAGCCAAGATGGTGAAGCAGA-3′ and 5′-GCTCCAGAAAATTCACCCCACC-3′, respectively. For NLRP3, the primer set was 5′-CGTCTTTGAGCCTTCTTGGTAG-3′ (forward) and 5′-GTCATTGTCCTGGTGTCTTCC-3′ (reverse). For IL-1β, the primer set was 5′-GATGGCTTATTACAGTGGCAATG-3′ (forward) and 5′-TAGTGGTGGTCGGAGATTCG-3′ (reverse). For caspase-1, the primer set was 5′-AGTCACACAAGAAGGGAGGAGA-3′ (forward) and 5′-TTCTTCCCAGGGACCTGTTCTT-3′ (reverse). ACTB (β-actin) was used as the housekeeping gene and amplified using the forward primer 5′-ACAGAGCCTCGCCTTTGC-3′ and the reverse primer 5′-ATCACGCCCTGGTGCCT-3′.

PCR amplification was performed on an automated PCR amplification system (Roche Diagnostics, Switzerland). Subsequently, gene expression levels were normalized to β-actin using the 2−ΔΔCt (fold change) formula. In addition, 2% agarose gel electrophoresis was used to confirm the size of PCR products by comparison with a 10kb DNA ladder marker.

Investigation of the protein levels of TXNIP and NLRP3 by western blotting

An immunoprecipitation method (RIPA) was used to extract protein from frozen PBMC samples using lysis buffer (Santa Cruz, USA). Briefly, the protein content of PBMCs was determined by homogenizing them for 5 minutes on ice in lysis buffer containing protease inhibitor (Sigma-Aldrich, USA) and phosphatase inhibitors (Sigma-Aldrich, USA). The supernatants were collected, shaken for two hours at 4°C, centrifuged at 12,000×g for 20 minutes (4°C), and finally stored at -20°C until further analysis.

Using the bicinchoninic acid protein assay (BCA), the total protein concentration was determined. Electrophoresis of 50 µg of each protein extract was performed on 10% SDS-PAGE, and then electroblotting was performed on UltraCruz® Nitrocellulose Pure Transfer Membrane (0.22 m) for one hour. After blocking with 5% nonfat dry milk (Merck, Germany), membranes were treated with primary antibodies, including polyclonal rabbit anti-TXNIP (STJ113636, St. John's Laboratories, UK), polyclonal rabbit anti-NLRP3 (STJ98852, St. John's Laboratories, UK), and anti-actin antibody (ab119716, Abcam, UK) and incubated overnight at 4°C. We used ab6721, an anti-IgG secondary antibody (Abcam, UK) for one hour at room temperature after washing the membranes with PBST and incubating them with horseradish peroxidase-labeled anti-IgG secondary antibodies (ab6721, Abcam). Immune reaction signals were visualized using a chemiluminescence kit (Bio-Rad, Germany). The relative expression levels of proteins were normalized using ImageJ software (https://imagej.nih.gov/ij).

Measurement of IL-1β protein by ELISA

IL-1β protein expression in PBMC samples was determined using the LEGEND MAX™ Human IL-1β ELISA kit (BioLegend, San Diego, USA), according to the manufacturer's instructions. The standard curve was utilized to quantify the IL-1β protein concentration in the samples, which was then represented in picograms per microgram of total protein (pg/µg).

In vitro culture of PBMCs

In the second phase of the study, PBMCs were isolated from a set of three independent healthy subjects. Cells were cultivated at a concentration of 3×10^6 PBMCs per well in one ml of culture media. Dulbecco's modified DMEM media with 20% FBS, 100 IU/ml penicillin, and 100 mg/ml streptomycin, supplemented with 0.25 mg/ml amphotericin, was used to cultivate the cells. Culture plates were then incubated at 37 °C in 5% CO_2, and once the confluency had reached 80-90%, cells were trypsinized, counted, and transferred into three sets of triplicate wells in 6-well plates at a concentration of 4×10^5 cells/ml. PBMCs were assigned as group 1 (Control), group 2 (Infected), and group 3 (Infected + treated with Vit. C). Cells from groups 2 and 3 were infected with the SARS-CoV-2 virus in Vero-E6 at passage 7, which was purchased from Pasteur Institute of Iran (Tehran, Iran) with a viral titer at 1×10^6 by TCID50 (Median Tissue Culture Infectious Dose). Cells in group 3 were then treated with a 10 mM solution of Vit C (21) for 4 hours and were used for further analysis as described below.

Infection of PBMCs with the SARS-CoV-2 virus

Cells from groups 2 and 3 were infected with the SARS-CoV-2 virus with 1 MOI (multiplicity of infection; the number of viral particles relative to host cells), according to the safety conditions and work protocols (22), and were placed in 5% CO_2 Memmert incubator (Memmert GmbH, Germany) at 37 °C. All experiments involving SARS-CoV-2 propagation were carried out in a Pasture Institute biosafety level 3 laboratory under the Class III laminar air flow cabinet (Airstream class III, Esco Lifesciences Group, Singapore).

Verification of PBMCs infection with the SARS-CoV-2 virus

To confirm infection of PBMCs with SARS-CoV-2 virus, an indirect immunofluorescence staining method was used (21). For this purpose, first, the virus-infected PBMCs of groups 2 (infected) and 3 (infected and treated with Vit C) were collected and centrifuged at 300×g for 5min. The supernatant was discarded, and 50 µl of concentrated PBMCs was transferred to slides coated with 0.1% polylysine (CITOGLAS, China), and the slides were incubated at 37°C for 40 min to allow cell adhesion. After that, the cells were fixed with 4% paraformaldehyde. To identify the cells infected with SARS-CoV-2, an anti-mouse spike antibody (Invitrogen Corporation, USA) was first added to the cells, and after washing with PBS, a conjugated polyclonal anti-mouse antibody was used as a secondary antibody (Thermo Fisher Scientific, A21202). Finally, the green fluorescent light emitted by virus-infected cells was detected using a UV microscope (Motic, USA). Although characterization of the infected cells based on cellular morphology is not easily applicable, the luminosity and shininess of emitted green light make infected cells distinct from non-infected cells with a very dim light.

Investigating the antioxidant effects of Vit. C on the PBMCs infected with the SARS-CoV-2 virus

Among three PBMC experimental groups, including group 1 (Control), group 2 (Infected), and group 3 (Infected + Vit. C), cells from the third group were treated with 10 mM Vit C solution for 4 h in 37°C to investigate the inhibitory effects of Vit C on virus-induced oxidative stress and inflammation in PBMCs. Then, in all 3 groups, the ROS content was determined by the DCFH-DA method, and TAC, TOS, and MDA levels were evaluated by appropriate KIAZIST kits. In addition, Western blotting was utilized to determine the protein levels of TXNIP and NLRP3, while the IL-1β protein level was measured using the Ebioscience ELISA kit (Thermo Scientific, Germany). All measurements in the second phase of the study (*in vitro*) were carried out with the same relevant methods as in the first phase of the study.

Statistical analysis

All statistical analyses were performed using SPSS Statistics software version 26.0 (SPSS Inc., USA). Descriptive analysis was reported in mean ± SEM, and $p < 0.05$ was considered statistically significant. The normality of variables was checked by the Shapiro-Wilk test. Comparison of variables with normal distribution between two groups was done with the Student's t-test, and the Mann–Whitney U test was used to compare variables without normal distribution. In the second phase of the study, a one-way ANOVA followed by a Post Hoc Tukey test was performed to compare differences in the means of variables. The correlation of variables was reported using the Spearman correlation test.

RESULTS

Oxidative stress increased in PBMCs of COVID-19 patients

Characteristics of the oxidative stress system, including ROS, TAC, TOS, MDA, and OSI (oxidative stress index calculated as TOS/TAC ratio), were determined to show any imbalance in cellular redox status. As shown in Figure 1, patients with COVID-19 showed a marked reduction in TAC (80.54 ± 4.64 *vs.* 47.25 ± 6.09 nmol/mg, $p < 0.05$) in PBMCs while significant increases were observed in ROS content (135.4 ± 15.02 *vs.* 212.9 ± 23.35 MFI, $p < 0.05$), in lipid peroxidation (17.55 ± 1.13 *vs.* 29.83 ± 1.6 nmol/mg, $p < 0.05$), and in TOS (14.78 ± 7.43 *vs.* 27.93 ± 2.11 nmol/mg, $p < 0.05$) compared with the control group. In addition, OSI, calculated as the TOS to TAC ratio, showed that in patients with COVID-19 is nearly six times higher (1.22 ± 0.35) than that of control (0.23 ± 0.02, $p < 0.0001$) subjects (Figure 1).

COVID-19 patients' PBMCs displayed an imbalance of the thioredoxin system

TXNIP expression was significantly higher in COVID-19 patients compared with the control group at both transcription (1.46 ± 0.07 *vs.* 0.95 ± 0.04, $p < 0.0001$) and protein level (1.80 ± 0.07 vs. 1.03 ± 0.04, $p < 0.05$) compared to the control group. As shown in Figure 2, comparison of COVID-19 patients with control subjects showed

significantly higher levels of TXNIP and significantly lower levels of TRX-1 (0.602 ± 0.04 vs. 1.006 ± 0.1, p = 0.0004, respectively).

The enhancement of TXNIP-activated NLRP3 inflammasome in PBMCs of COVID-19 patients

An increased gene expression and protein levels of TXNIP led to the simultaneous significant ($p < 0.0001$) increases in the expression of NLRP3, caspase-1, and IL-1β genes in PBMCs of COVID-19 patients compared to control subjects (Figure 3). Interestingly, the increment in the expression of NLRP3 and caspase-1 genes was followed by a significant ($p < 0.0001$) enhancement in the NLRP3 protein level, which in turn led to the rocketing of IL-1β protein levels ($p < 0.05$) in COVID-19 patients, as shown in Figure 3. Our results showed that an increase in TXNIP transcription and protein level (as observed in Figure 1) triggered a cascade of induction in gene expression and/or protein levels of NLRP3, caspase-1, and IL-1β, which is characteristic of activation of the NLRP3 inflammasome.

There was a similar increase in the mRNA expression of NLRP3 in the patient group compared to the control group (1.62 ± 0.093 vs. 0.96 ± 0.03, $p < 0.0001$) and IL-1β (1.69 ± 0.082 vs. 1.003 ± 0.068) and protein levels of NLRP3 (1.7 ± 0.07 vs. 0.97 ± 0.05, $p < 0.0001$), IL-1β (24.43 ± 0.87 vs. 9.79 ± 0.38 pg/ml, $p < 0.0001$) were obtained. Interestingly, IL-1β protein increased significantly (Figure 3) with the increase of caspase-1 in the gene expression level of the patient group compared to the control group (1.51 ± 0.08 vs. 0.95 ± 0.13, $p < 0.0001$).

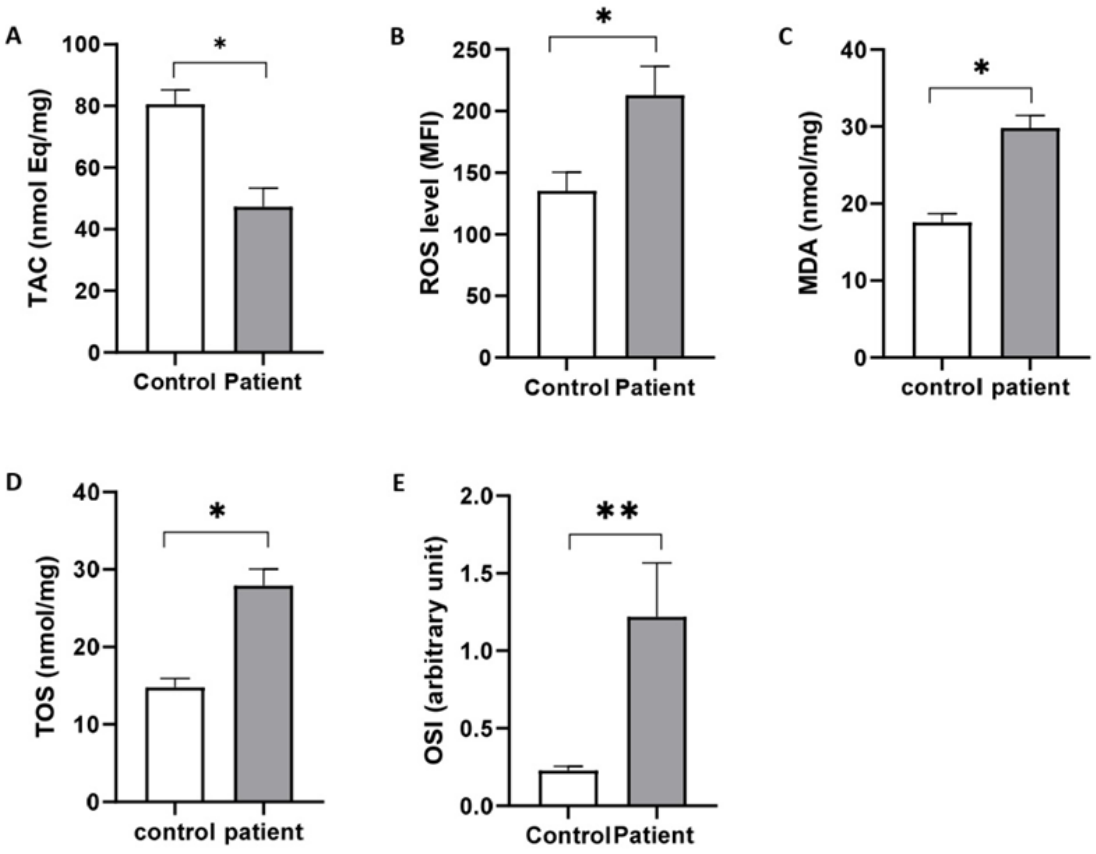

Figure 1. Determination of parameters involved in the oxidative stress system in PBMCs of healthy subjects and COVID-19 patients. (A) Total antioxidant capacity, (B) reactive oxygen species level, (C) malondialdehyde (MDA) level as an indicator of lipid peroxidation, (D) total oxidative status, and (E) the oxidative stress index (OSI) calculated as TOS to TAC ratio. MDA: Malondialdehyde, MFI: Mean fluorescence intensity, OSI: oxidative stress index, ROS: Reactive oxygen species, TAC: Total antibody capacity, TOS: Total antioxidant status. Asterisk (*) indicates $p < 0.05$ and (**) $p<0.0001$

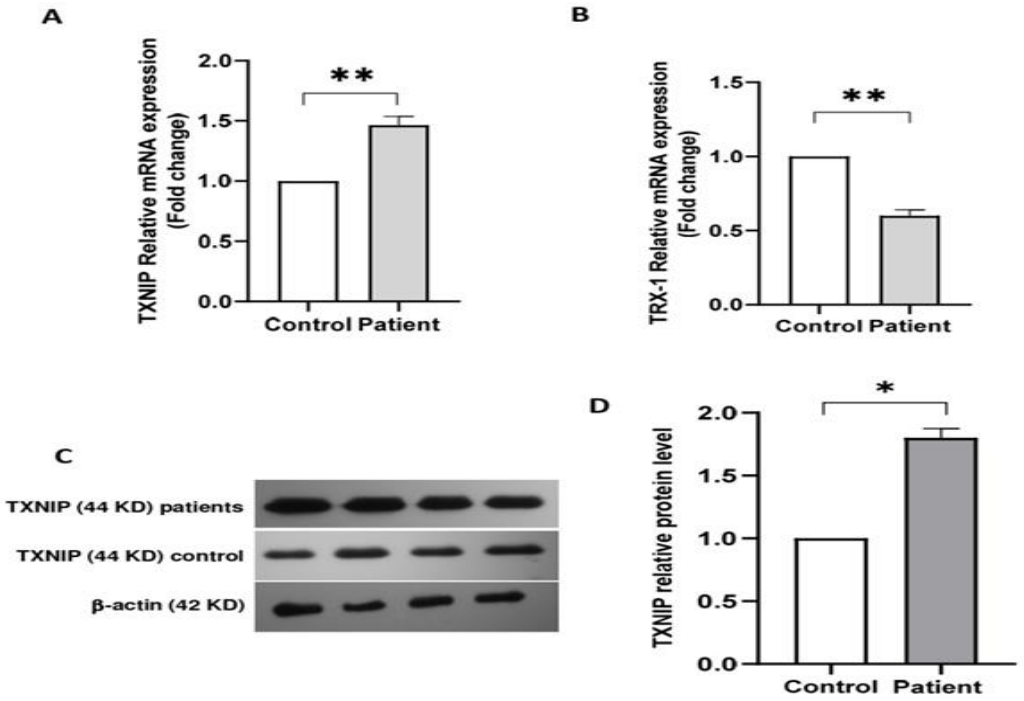

Figure 2. The expression levels of the thioredoxin system in PBMCs of patients with COVID-19 and healthy controls, as determined by RT-qPCR and western blotting techniques. (A) TXNIP gene expression, (B) TRX-1 gene expression level, (C) The results of western blotting for TXNIP and internal standard β-actin, and (D) TXNIP protein level measured by densitometric method using ImageJ software. TRX-1: thioredoxin-1 and TXNIP: thioredoxin interacting protein. Results are reported as mean ± SEM. Asterisk (*) indicates $p < 0.05$ and (**) $p<0.0001$

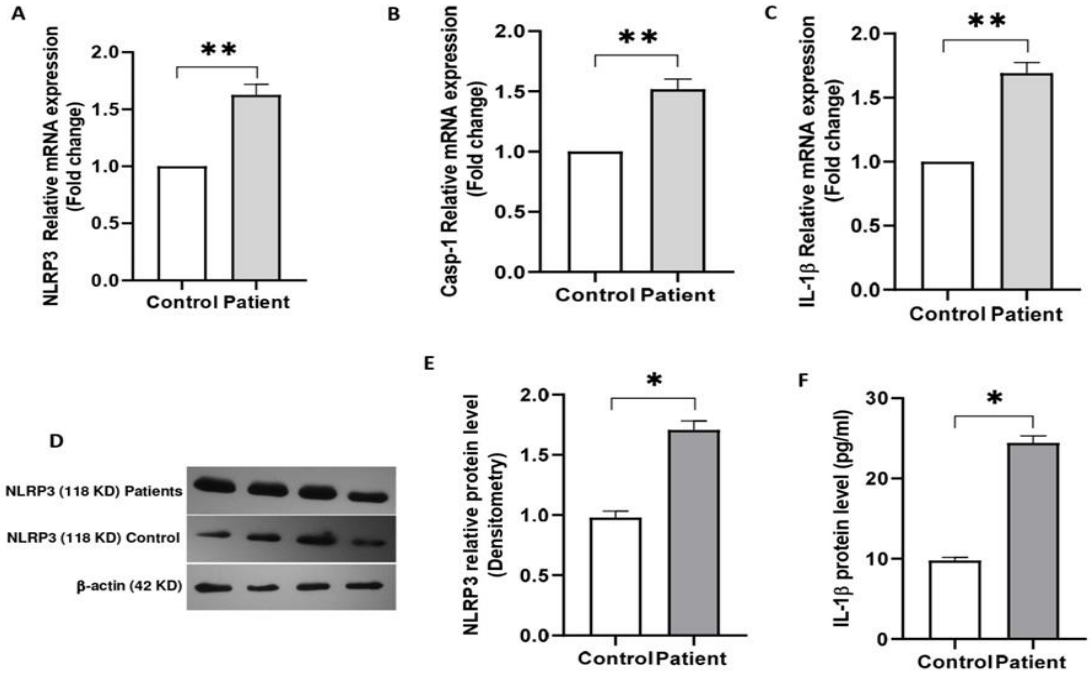

Figure 3. The gene expression and protein levels of the members of the NLRP3 inflammasome complex, as determined by RT-qPCR, western blotting, or ELISA, as appropriate. (A) mRNA expression of NLRP3 gene, (B) CASP-1 gene expression level, (C) IL-1β gene expression level, (D) the results of western blotting for NLRP3 and internal standard β-actin, (E) NLRP3 protein level measured by densitometric method using ImageJ software, and (F) IL-1β protein level determined by ELISA. Data are reported as mean ± SEM and aterisk * and ** represents $p < 0.05$ and $p<0.0001$, respectively

Cross-talk between oxidative stress, TXNIP/TRX-1 system, and NLRP3 inflammasome in COVID-19 patients

Increased ROS content in PBMCs was correlated with the enhancement in the production of IL-1β, as shown in Table 1. As it was initially hypothesized, the overproduction of IL-1β was mediated through cross-talk between the TXNIP-TRX system and NLRP3 inflammasome since a strong correlation was observed between TXNIP and NLRP3 protein levels (R=0.631, p<0.01). A positive association was also observed between NLRP3 and IL-1β, indicating activation of the NLRP3 inflammasome, leading to the production of IL-1β (Table 1).

Table 1. Correlations between variables in COVID-19 patients, as determined by Spearman Analysis

Correlation	Spearman correlation coefficient (R)	P value
ROS - IL-1β	0.329	0.01
TXNIP – NLRP3	0.631	0.01
TXNIP - IL-1β	0.607	0.01
NLRP3 - IL-1β	0.625	0.01

IL-1β: interleukine-1 beta, NLRP3: nucleotide-binding oligomerization domain receptors containing pyrin domain 3 (NLRP-3) inflammasome, ROS: reactive oxygen species, TXNIP: thioredoxin interacting protein.
ROS content is given in MFI units, while all other data are represented in the table, indicating protein levels in PBMCs

The observation of a substantial connection (R=0.607, p<0.01) between the amount of TXNIP protein and IL-1β production further supports the competency of the cross-talk between the TXNIP-TRX system and the NLRP3 inflammasome. The positive correlation between IL-1β protein expression was more related to NLRP3 and CASP1 gene expression (R=0.566, R=0.481, p<0.01). Due to TAC negative regulation, we observed the most negative correlation between TAC-NLRP3 gene expression (R=-0.445, p<0.01) and IL-1β protein expression (R=0.441, p<0.01), as indicated in Table 2.

In vitro results

Infection of human PBMCs with SARS-CoV2

Since human lymphocytes and monocytes are prone to SARS-CoV-2 infection in laboratory conditions, PBMCs isolated from three independent healthy individuals were first cultured with normal DMEM media. PBMCs were examined under a microscope after 8 h to ensure cell viability. PBMCs were then infected with SARS-CoV2 virus at MOI=1 and incubated for 6 hours. Cells were centrifuged, supernatants discarded, and the cells were collected. The infection results were confirmed by immunofluorescence microscopy analysis. The presence of a viral infection was confirmed by binding a specific fluorescent antibody to the virus particles, followed by the observational analysis under a UV microscope. The green fluorescent light was emitted by infected cells, while no emission was detected in uninfected cells, as well as infected cells treated with vitamin C (Figure 4).

Table 2. Correlations between variables in COVID-19 patients, as determined by Spearman Analysis

Correlation	Spearman correlation coefficient (R)	P value
NLRP3(GE) - IL-1β (PL)	0.566	0.01
CASP1(GE) - IL-1β (PL)	0.481	0.01
NLRP3 (GE) - TOS	0.448	0.01
NLRP3 (PL) - TOS	0.401	0.01
TXNIP (PL)- TOS	0.494	0.01
IL-1β (PL) - TOS	0.490	0.01
NLRP3 (GE) - MDA	0.461	0.01
CASP1 (GE) - MDA	0.406	0.01
NLRP3 (PL) - MDA	0.490	0.01
TXNIP (PL)- MDA	0.541	0.01
IL-1β (PL) - MDA	0.507	0.01
CASP1 (GE) - ROS	0.334	0.01
NLRP3 (GE) - TAC	-0.445	0.01
NLRP3 (PL) - TAC	-0.353	0.01
TXNIP (PL)- TAC	-0.388	0.01
IL-1β (PL) - TAC	-0.441	0.01

CASP1: caspase-1, IL-1β: interleukine-1 beta, NLRP3: nucleotide-binding oligomerization domain receptors containing pyrin domain 3 (NLRP-3) inflammasome, ROS: reactive oxygen species, TAC: total antioxidant capacity, TOS: total oxidant status, TXNIP: thioredoxin interacting protein, GE: gene expression level, PL: protein level
The numbers refer to the correlation coefficient (R). The p-value represents significant correlations at the 0.01 level (2-tailed).

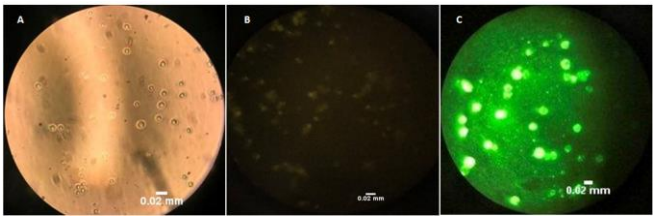

Figure 4. Infection of PBMCs with the SARS-CoV-2 virus, as verified by the addition of cell surface-specific antibodies. (A) PBMCs without infection with the virus, (B) non-infected PBMCs treated with primary and secondary antibodies, (C) PBMCs infected with SARS-CoV-2 virus and treated with primary and secondary antibodies. PBMCs were cultivated and stained with a primary anti-mouse spike antibody and a polyclonal conjugated anti-mouse antibody as the secondary antibody. The infected PBMC cells emitted green fluorescent light due to the antibody-conjugated effects. Scale bar shows 0.02 mm, and the magnification of the microscopy images is ×400

Vitamin C treatment restored redox status in infected PBMCs

Biomarkers of the cellular redox system, ROS, MDA, TAC, TOS, and OSI, were measured in all three groups of cultured PBMCs. As shown in Figure 5, the ROS content, MDA level, TOS, and OSI were significantly increased in infected PBMCs, whereas infection of the PBMCs with SARS-CoV-2 virus significantly lowered the TAC level compared with control PBMCs. However, treatment of the infected cells with Vitamin C completely suppressed any inclination in oxidative stress factors (Figure 5). After treatment of the infected PBMCs with Vitamin C, the amount of the ROS, MDA, TOS, and OSI remarkably declined in such a way that no significant differences were observed between control and treated cells in ROS, TOS, and OSI. Treated cells even had a higher level of antioxidant capacity (TAC) than control cells (Figure 5).

Vitamin C treatment suppressed production of IL-1β by downregulating TXNIP level and inhibiting activation of NLRP3 inflammasome in infected PBMCs

Western blot analysis revealed that PBMCs had significantly higher amounts of TXNIP, NLRP3, and IL-1β proteins following SARS-CoV-2 virus infection (Figure 6). TXNIP, NLRP3, and IL-1β levels of infected cells were almost 2.6-fold, 2.2-fold, and 2.6-fold higher than the corresponding levels in control PBMCs (Figure 6). However, treatment of the infected PBMCs with Vitamin C in group 3 sharply lowered TXNIP, NLRP3, and IL-1β protein levels compared with untreated infected PBMCs in group 2. The suppressing effect of Vit. C on TXNIP and NLRP3 expression and its inhibitory effect on IL-1β production were such that no significant differences were observed in TXNIP, NLRP3, and IL-1β protein levels between treated and control PBMCs (Figure 6).

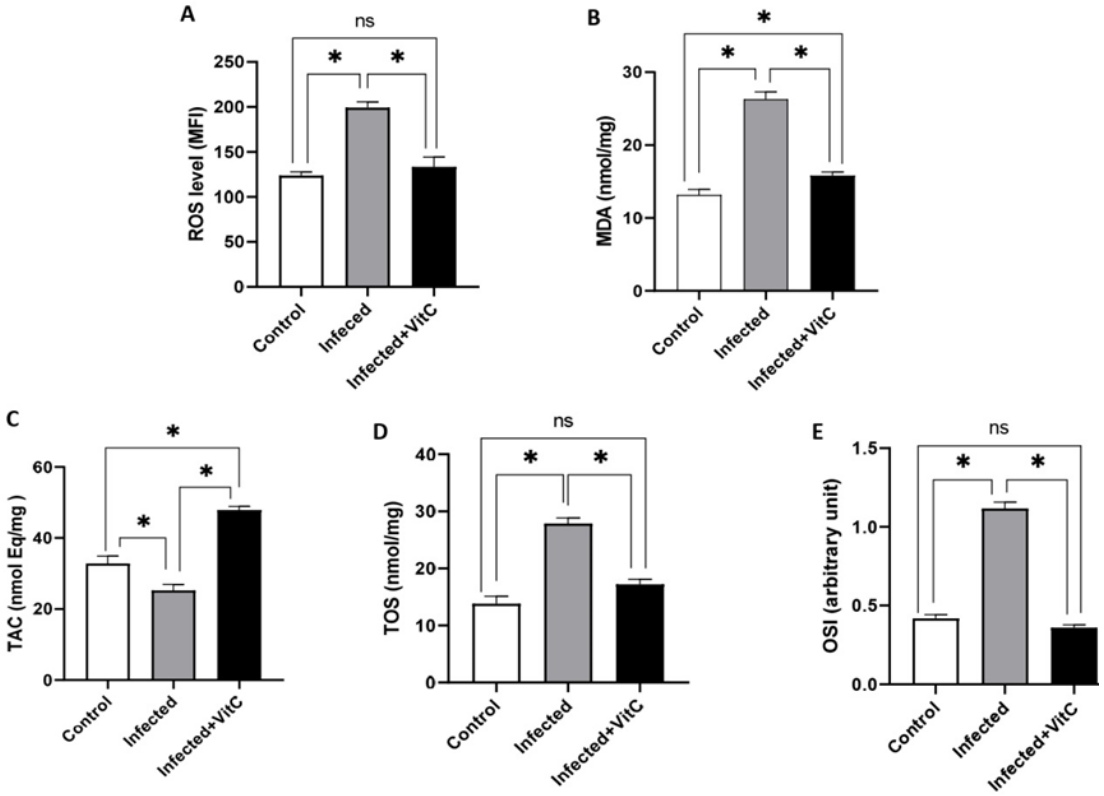

Figure 5. Protective antioxidant effects of vitamin C on the PBMCs infected with the COVID-19 virus compared to uninfected cells. (A) The intracellular level of ROS in PBMCs, (B) malondialdehyde (MDA) level as an indicator of lipid peroxidation, (C) total antioxidant capacity, (D) total oxidative status, and (E) the oxidative stress index (OSI) calculated as TOS to TAC ratio. MDA: Malondialdehyde, MFI: Mean fluorescence intensity, OSI: oxidative stress index, ROS: Reactive oxygen species, TAC: Total antibody capacity, TOS: Total antioxidant status. Asterisk (*) represents $p<0.05$

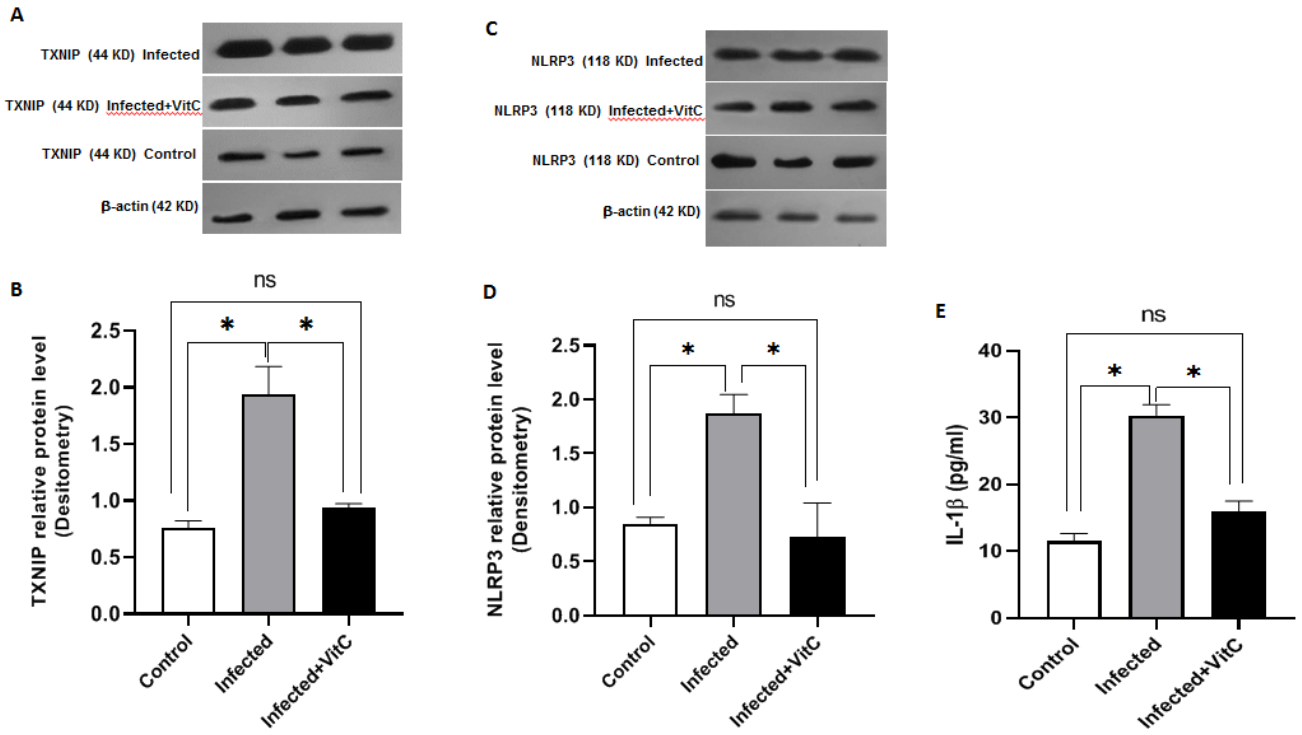

Figure 6. Protective effects of vitamin C in ameliorating the thioredoxin system and preventing activation of NLRP3 inflammasome complex in the PBMCs infected with the COVID-19 virus compared to uninfected cells. (A) The results of western blotting for TXNIP and internal standard β-actin, and (B) TXNIP protein level measured by densitometric method using ImageJ software, (C) the results of western blotting for NLRP3 and internal standard β-actin, (D) NLRP3 protein level measured by densitometric method using ImageJ software, and (E) IL-1β protein level determined by ELISA. Results are reported as mean ± SEM, and an asterisk (*) represents $p < 0.05$. ns: not significant

DISCUSSION

COVID-19 was introduced as a lethal disease worldwide, and despite significant recent medical, technological, and therapeutic advances, the disease is responsible for more than 6 million deaths (14). According to studies, oxidative stress may be a significant factor in viral infections, particularly coronavirus infections like SARS-CoV and SARS-CoV-2. In summary, the recent research (23) suggests that the absence or reduction of oxidative stress would significantly impact the initial phase of viral infection. Therefore, understanding the role of oxidative stress in COVID-19 infection, including the functions of antioxidants and redox proteins, could assist in orienting possible therapeutics.

The initiation of COVID-19 is due to oxidative damage caused by the production of ROS, inflammation, activation of inflammasomes, and cytokine storms (24). Increased ROS and subsequent oxidative stress caused by viral infection create a favorable environment for inflammation (7). Accordingly, the present study comparatively measured the characteristics of oxidative stress, including ROS, TAC, TOS, MDA, and OSI, as well as the expression of TXNIP, TRX, NLRP3, caspase-1, and IL-1β levels at the transcriptional and/or protein levels in PBMCs of healthy subjects and COVID-19 patients. In addition, to confirm the hypothesis that oxidative stress may increase expression of TXNIP and lead to the activation of NLRP3 inflammasome and production of IL-1β, in the second phase of the study, PBMCs were infected with SARS-CoV-2 virus, and the beneficial inhibitory effects of Vitamin C, as a potent antioxidant, on the cross-talk between TXNIP and activation of NLRP3 inflammasome and production of IL-1β were investigated in vitro.

This study revealed that COVID-19 patients had lower levels of TAC but higher levels of ROS, MDA, and TOS than the healthy group. In COVID-19 patients, serum

levels of TAC, ROS, and MDA were evaluated in previous studies (18). On the other hand, after the increase of ROS through oxidative stress during viral infections, inflammatory pathways are activated, which causes cytokine production and tissue destruction (9). Limited experimental data elucidate the mechanisms involved in the induced inflammatory responses in immune cells during the SARS-CoV-2 cytokine storm. IL-1β is a form of interleukin-1 made primarily by macrophages and helps lymphocytes fight infections. In the present study, we observed overexpression of IL-1β in patients with COVID-19 compared to healthy individuals. These findings were consistent with those of Valizadeh et al., who reported the increase of IL-1β in COVID-19 patients (10).

Patients with COVID-19 may be affected by inflammation caused by TXNIP/TRX, NLRP3, and inflammasomes. Peroxisomes are formed by TRX-1 and TXNIP, which play an important role in regulating oxidative stress-related diseases and redox status in cells (16,20). The increase of ROS during COVID-19 infection occurs through the dysfunction of the oxidative stress system and the activation of the NLRP3 inflammasome pathway, and the initiation of a cytokine storm (18, 25). Oxidative stress tends to release TXNIP to interact with NLRP3; when the NLRP3 is activated, its procaspase-1 transforms into the active effector protease caspase-1 that causes the maturation and cleavage of pro-inflammatory cytokines, including pro-interleukin-1β (23, 26). Caspase-1 activation, IL-1β production, and pyroptosis have been observed in human monocytes, both in infected ICU patients and in vitro SARS-CoV-2 infected monocytes (15, 27). The arrestin-like domain of TXNIP regulates protein-protein interaction by dissociating from TRX-1 under ROS conditions (28). As a result of binding to and activating NLRP3, TXNIP causes this protein to oligomerize and form an inflammasome complex (18). Cytokines that promote inflammation are produced by the NLRP3 inflammasome. Therefore, high levels of TXNIP are likely to be associated with inflammation induced by increased NLRP3 (14, 29). Previous studies have shown the overexpression of NLRP3 in COVID-19 patients (30) and its role in accelerating the process of cytokine storm (31). In the current investigation, COVID-19 patients had elevated levels of NLRP3 gene expression in conjunction with elevated IL-1β mRNA and protein levels as the final product of NLRP3 inflammasome activation in PBMC cells compared to the healthy group. These findings are consistent with the results of the study by Houshmandfar et al., which showed that the expression of NLRP3 and IL-1β genes was higher in COVID-19 patients (31). Although NLRP3 overexpression was previously evaluated in COVID-19 patients, the regulation of TRX-1 in PBMCs is still unclear. In the present study, the expression level of the TRX-1 gene was evaluated in PBMCs to better understand the onset of cytokine storm. The results showed that the expression level of the TRX-1 gene in PBMCs of COVID-19 patients was significantly lower than that of the healthy group. In addition to its reducing effect on disulfide bonds, TRX-1 is an antioxidant that prevents oxidative stress-induced cellular damage. As part of COVID-19 pathogenesis, TRX-1 plays an important role due to its ROS-inhibitory effect (17,28). TRX-1 can be synthesized ubiquitously, and under certain circumstances, it can be secreted from cells, explaining the controversial concentrations (30). Several hormones and natural metabolic substances can influence circulating levels of TRX-1, including cAMP, prostaglandins, hemin, and estrogen (31, 32). In the present study, it was discovered that TRX-1 level and redox power were reduced because of the disruption of TRX-1/TXNIP interactions. This molecule is believed to play a crucial role in the development of oxidative stress in COVID-19. TRX-1 expression and function are negatively regulated by TXNIP by forming a disulfide-linked complex with reduced TRX-1 (TXNIP/TRX-1) (27, 33). The activation of TRX-1 in COVID-19 patients is thought to occur when TXNIP dissociates from TRX-1 under stress conditions after TXNIP's inhibitory effect is eliminated. Overexpression of TXNIP has been shown to suppress TRX-1 expression. COVID-19 patients' PBMCs were significantly higher than healthy group PBMCs in terms of TXNIP mRNA and

protein levels, according to the present study. Accordingly, the decrease in TRX-1 expression is caused by an increase in TXNIP mRNA or protein levels in patients with COVID-19. This situation leads to the exacerbation of the disease of COVID-19. Moreover, TXNIP levels were directly correlated with ROS, TOS, and MDA levels, while TAC levels were inversely correlated. The increase of TXNIP and the decrease of TRX-1 levels, the imbalance in the oxidative status of oxidation, and the strong correlation observed between these factors in COVID-19 patients may indicate the possible occurrence of oxidative stress in these patients. However, research on the role of the thioredoxin system in COVID-19 patients requires more study, such as finding other components of the thioredoxin system. COVID-19 shows TXNIP to be an important mediator of ROS and inflammation among various factors that can initiate inflammatory pathways (16). To prove this, PBMCs cells were cultured, and the expression level of IL-1β, NLRP3, and TXNIP proteins in the plate not infected or treated with vitamin C was significantly different from the other two groups. Vitamin C has an anti-inflammatory effect and therefore has a therapeutic role in treating systemic inflammatory disease mediated by inflammation-dependent pyroptosis and pro-inflammatory cytokines such as IL-1β (31).

In the current investigation, we also showed that inflammation was caused by IL-1β upregulation, and NLRP3 gene expression correlated positively and significantly with IL-1β levels. Also, IL-1β and NLRP3 had the highest sensitivity and specificity in PBMCs of patients compared to control groups. It was also verified using a treatment with vitamin C as an antioxidant. It affects the downregulation in IL-1β, NLRP3, and TXNIP protein expression, and also the decrease of ROS, TOS, and MDA levels. The activation of NLRP3 may be involved in inflammation and the initiation of cytokine storms in COVID-19 patients. Our results should be interpreted with the following implications in mind: 1) possible effects of ethnicity, gender, and being old or young on biochemical parameters; 2) A further investigation of NLRP3 inflammasome components was not carried out in this study; and 3) our small sample size.

CONCLUSION

Activation of NLRP3 inflammasomes, caspase-1 production, and inflammation in COVID-19 patients may be associated with the thioredoxin system (TXNIP/TRX-1) in PBMCs. Considering the beneficial effects of vitamin C, as observed in the second phase of the present study, the use of Vit. C as an adjunctive therapeutic strategy is recommended in the treatment of COVID-19 patients.

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Conflict of interest

The authors declare no conflict of interest.

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