Original Article

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TANAFFOS

Glutathione Peroxidase (GPx) and Glutathione Reductase Gene Expression as Key Factors in the Glutathione System, in Oxidative Stress Pathways in Patients with COVID-19

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Background: COVID-19 emerged as a major global health challenge, rapidly spreading worldwide and affecting millions of people. In response to the COVID-19 pandemic, this study aimed to address the gene expression of Glutathione Peroxidase (GPx) and Glutathione Reductase at the mRNA level and specify which group is being referenced.

Materials and Methods: RNA was extracted from blood samples of COVID-19 patients admitted to the ICU with severe symptoms of the disease, as well as infectious wards, with mild symptoms of COVID-19, and then cDNA was synthesized. The sequence of the designed primers was examined in the human genome sequence using BLAST software, and finally, the Real-Time RT-PCR reactions were duplicated.

Results: Glutathione Peroxidase and Glutathione Reductase markers in the peripheral blood of patients with severe and mild symptoms showed a statistically significant difference in terms of positivity (P-value <0.001). Then, the Ct of each sample was determined. The difference in the expression of markers between the two groups was calculated using "$2^{-\Delta\Delta Ct}$" and showed that the increased expression of these markers in the group of patients with severe symptoms compared to mild ones.

Conclusion: Our findings suggest that glutathione peroxidase (GPx) and glutathione reductase (GR), key components of the glutathione antioxidant system, may be upregulated in response to increased reactive oxygen species (ROS) levels and oxidative stress.

Keywords: Glutathione Peroxidase; Glutathione Reductase; Oxidative Stress; Real-Time-PCR

INTRODUCTION

Respiratory viral infections, including COVID-19, refer to a group of diseases that have recently emerged as a new challenge in the medical sciences, involving millions throughout the world (1, 2).

On March 11, 2020, the World Health Organization declared COVID-19 a pandemic disease because it spread rapidly around the world, showing a high rate of human-to-human transmission (3). There is a relationship between the age, severity, and mortality of SARS-CoV-2 infection, i.e., COVID-19 disease (2).

Although COVID-19 infection is usually asymptomatic or mild, people who are at a higher risk for the disease have certain risk factors, such as smoking, male gender, hypertension, heart disease, obesity, asthma, cancer, immunosuppression, as well as chronic kidney and liver disease (4-7).

Respiratory viruses, including influenza (IV), human rhinovirus (HRV), parainfluenza, adenovirus, and coronavirus (CoV), can infect the human upper and/or lower respiratory tract (8).

Patients with COVID-19 have a variety of signs and symptoms, from asymptomatic patients with mild colds to significant changes in clinical and laboratory examinations, which can lead to severe organ failure and death (9, 10).

Respiratory viral infections are associated with the production of cytokines, inflammation, cell death, and other pathophysiological processes, which may be associated with redox imbalance or oxidative stress (OS) (11).

In general, free radicals are highly reactive molecules or atoms containing one or more unpaired electrons, which can induce oxidative damage to cells and tissues. These molecules, at physiological concentrations, are essential for normal cell function, while their high concentrations could lead to oxidative stress (12).

Due to the lack of stability, these molecules have a very high tendency to react with other molecules, thus damaging lipids, proteins, and even DNA, and can cause cell damage (12).

The antioxidant system of many products includes enzymes such as Superoxide Dismutase, Catalase, Glutathione Peroxidase-GPX, and Glutathione Reductase-GR, which act as neutralizers of oxygen forms and play a vital role in maintaining cell homeostasis (13-15).

The glutathione system plays a vital role in protecting the body from oxidative stress. This system converts hydrogen peroxide resulting from the activity of superoxide dismutase enzyme on superoxide ion, which is a dangerous reactive oxygen species (ROS), into water (16).

Overproduction of ROS and lack of antioxidant mechanisms are essential for virus replication and the resulting virus-associated disease (11).

Given the widespread impact of coronavirus disease 2019 (COVID-19) and its associated systemic effects, this study investigated the mRNA expression levels of glutathione peroxidase (GPx) and glutathione reductase (GR) as potential indicators of oxidative stress and increased reactive oxygen species (ROS) levels.

MATERIALS AND METHODS

Preparation and collection of samples

The project began with the design of a case-control study. Samples of 60 patients from the ICU and infectious wards were collected in April 2021. After obtaining informed consent and approval from the institutional ethics committee (IR.SBMU.NRITLD.REC.1399.198), blood samples were collected from 60 patients with COVID-19 admitted to Masih Daneshvari Hospital, including 30 patients with severe disease admitted to the intensive care unit (ICU) and 30 patients with mild disease admitted to the infectious diseases ward.

RNA extraction

Total RNA was extracted using the Qiagen RNA extraction kit according to the manufacturer's instructions. The concentration and purity of the extracted RNA samples were assessed using a BioTek NanoDrop spectrophotometer by measuring absorbance at 260 nm and calculating the A260/A230 ratio.

Synthesis of cDNA

The extracted RNA samples were used for cDNA synthesis according to the manufacturer's instructions provided with the Vivantis kit.

The website www.ncbi.nlm.nih.gov was used to design the primers. However, the designed primers using BLAST software were examined in the sequence of the human

genome; in this way, the characteristics of the sequences and the specificity of the binding point of the primers on the gene are ensured. In this study, the 18srRNA was used as a reference gene. The genes used and their primers are shown in Table 1.

Real-time PCR

The gene quantification methods were performed using the Real-Time PCR model ABI 7300 (Applied Biosystems, Foster City, CA, USA).

The volume of the reaction mix consisted of 3 µl of cDNA, 5 µl of SYBR-Green PCR reaction mixture (SYBR-Green PCR Master Mix), 2 µl of each forward and reverse primer, and 10 µl of nuclease-free deionized distilled water.

Moreover, the device's heat-time schedule for gene amplification includes initial DNA opening at 95° C for 30 seconds, and denaturation at 95 ° C for 20 seconds by repeating 40 cycles, binding primers to the template DNA at 62 °C for 60 seconds, and elongating the template strand over 30 seconds at 72 °C.

After the Real-time PCR reaction, the threshold difference (Ct) between the two groups of patients with severe and mild symptoms was obtained. Fold change is obtained using the formula $2^{-\Delta\Delta Ct}$.(17-22)

Statistical Analysis

The research data were statistically analyzed using SPSS 20. The t-test was used for statistical data analysis. The significance level was $P \leq 0.05$.

RESULTS

Samples were collected from two groups. Thirty patients with COVID-19, who were admitted to the ICU due to severe symptoms, and 30 patients with mild symptoms, who were admitted to the infectious wards.

Patients' symptoms are summarized in Table 2 based on the disease severity.

19 men and 11 women, and 16 men and 14 women were in the group of patients with severe and mild symptoms, respectively. The two groups were matched on age variables and compared on mean age using a t-test. In the group with severe symptoms, the age range was 28-65 years, with an average of 44.35 years, and in the group with mild symptoms, the age range was 26-64 years, with an average of 43.88 years. There was no significant difference in mean age between the two groups (P = 0.404), suggesting that age was unlikely to have confounded the comparison between the groups. Demographic information (patient specification) is shown in Table 3.

Table 2. Criteria for the severity of the disease in people with COVID-19 (23, 24)

Disease severity	Specifications
Mild	-SpO2≥94% on room air - Without hypoxia
Moderate	-SpO2=90-93% on room air Or - 24<respiratory rate<30 breaths/min Or - Pulmonary infiltration<50%
Severe	-SpO2<90% on room air Or - Respiratory rate>30 breaths/min Or - Pulmonary infiltration>50%
Critical	-SpO2<90% on room air - Requires oxygen via HFNC/NIV/ mechanical ventilation or - Pulmonary infiltration>50% or - With hemodynamic disorders/multi-organ failure/coagulation disorders usually high levels of IL-6

Table 1. Sequence and specifications of the used primers

	Glutathione peroxidase	Glutathione reductase	18srRNA
Forward primer	AGAAGTGCGAGGTGAACGGT	CCTTCTTTGGTGGCAATTCTATCT	GTAACCCGTTGAACCCCATT
Length	20	24	20
Reverse primer	CGATGTCAATGGTCTGGAAGC	CCCCTTCAGAGCCCATAGTCA	CCATCCAATCGGTAGTAGCG
Length	21	21	20
Product length	228	244	152
Optimal Temperature Annealing	59	60	56

Table 3. Clinical characteristics and demographic information of patients with COVID-19

Property	Total patients	Group	N	Mean	Std. Deviation	P
Age	60	Ward	30	43.88	9.5	0.404
		ICU	30	44.35	10.4	
Female	25	Ward	14	-	-	0.00
		ICU	11			
Male	35	Ward	16	-	-	0.00
		ICU	19			
Diabetes	6	Ward	2	-	-	0.001
		ICU	4			
Immune-deficiency Disease	2	Ward	-	-	-	0.00
		ICU	2			
Fever	24	Ward	8	-	-	0.00
		ICU	16			
Cough	21	Ward	7	-	-	0.001
		ICU	14			
Fatigue	22	Ward	7	-	-	0.00
		ICU	15			
Real-time PCR	57	Ward	28	-	-	0.00
		ICU	29			
IL-6	60	Ward	30	13.8	16.4	0.282
		ICU	30	8.8	5.1	
CRP	58	Ward	28	-	-	0.00
		ICU	30			

All Real-Time RT-PCR reactions were duplicated. The results were interpreted considering the melting curve. By evaluating the specific peak of the studied product, the specificity of Real-Time PCR amplification was confirmed.

Analysis of Studied Biomarkers' Expression

Glutathione Peroxidase and Glutathione Reductase markers in the peripheral blood of patients with severe symptoms were positive in 26/30 and 24/30, respectively. The positivity rates of these markers in the group of patients with mild symptoms were 21/30 and 19/30, respectively. Statistical comparison of the positivity of these markers in the two groups was performed using the Two-sample binomial test, which suggested a statistically significant difference between the two groups (P-value <0.001) (Figure-1).

Fold change calculation

First, the Ct of each sample is determined. The relative difference between the expressions of the marker in the two groups was calculated. Using the formula $2^{-\Delta\Delta Ct}$ for Glutathione Peroxidase and Glutathione Reductase showed that the expression of these markers in the group of patients with severe symptoms, compared to patients with mild symptoms, will increase: 1.48 and 1.32, respectively (Figure 2).

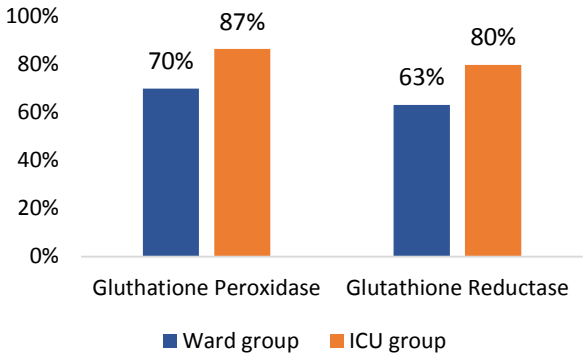

Figure 1. Glutathione Peroxidase and Glutathione Reductase positivity in patients with severe and mild symptoms

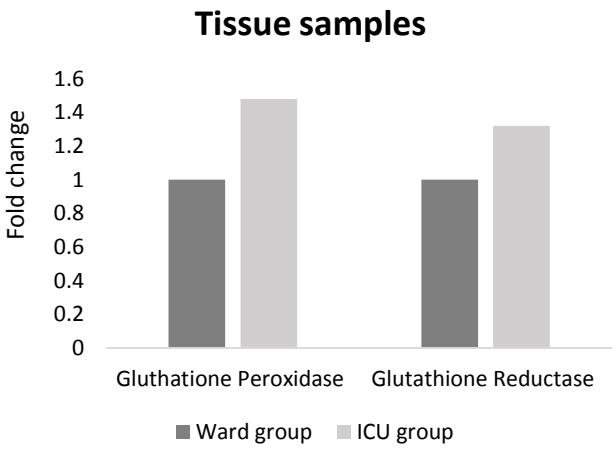

Figure 2. Difference of Glutathione Peroxidase and Glutathione Reductase in patients with severe symptoms compared to patients with mild symptoms

DISCUSSION

In general, there is a clear correlation between Oxidative Stress markers and the severity of many viral diseases, but SARS-CoV clinical data are limited. However, accumulating preclinical evidence suggests that excessive ROS production and impaired antioxidant defenses play important roles in the pathogenesis of SARS-CoV infection

and contribute to the progression and severity of respiratory disease. Studying animal models of the severe acute respiratory syndrome has shown increased ROS levels and impaired antioxidant defense during SARS-CoV infection (25).

A notable feature of COVID-19 is the significantly elevated levels of circulating cytokines and chemokines. Cytokine storm creates a pro-inflammatory environment, which leads to severe tissue damage and is associated with fatal outcomes in patients with COVID-19. The relationship between inflammation and OS is well established (6, 26).

Inflammation is an important factor in the cytokine storm (27). Accumulating evidence suggests that reactive oxygen species (ROS) can function as signaling molecules and important mediators of inflammatory responses. Moreover, the NF-κB transcription level is increased. NF-κB, in turn, is activated by ROS; thus, inflammation, directly or indirectly, results in increased ROS (28-30).

Not only is glutathione abundant in most cells, but it is also the most abundant antioxidant in the airway epithelial fluid, acting as a vital intracellular and extracellular antioxidant, protecting against oxidative stress and reducing inflammatory processes in the lungs (31).

The glutathione system plays an important role in dealing with oxidative stress. Glutathione peroxidase and glutathione reductases are critical enzymes in this cycle, and the expression of their genes increases as a result of the rise in ROS and exposure of the body to oxidative stress. Oxidative stress induced by reactive oxygen species induces and destroys apoptosis in cells (16).

Glutathione (GSH) has the main antioxidant function in all tissues. The high concentration of its reduced form shows its major role in controlling many processes, including detoxification, antiviral defense, and immune response (32).

Glutathione can prevent damage to important ROS-induced cellular components and their derivatives, including free radicals, peroxides, lipid peroxides, or organic pollutants and heavy metals (33).

Shao et al. showed that there is upregulation of mitochondrial genes and genes responding to oxidative stress in peripheral blood mononuclear cells (PBMC) in patients recovering from SARS-CoV (34).

Lin et al. showed that viral protease in SARS-CoV significantly increased ROS production, which in turn stimulated apoptosis and antioxidant factors (35).

Another study showed that the SARS-CoV viral protease activates the NF-κB pathway, which is associated with increased ROS levels in cells, which promotes apoptosis-related genes and proinflammatory genes. Therefore, it is suggested that the viral protease in SARS-CoV stimulates RF-activated NF-κB signal, and may play a key role in the pathophysiology of SARS-CoV(2).

GSH plays a major role in the pathophysiology of human disease (39 of glutathione-1); GSH imbalance is observed in a wide range of pathological conditions, including lung infections, HIV, diabetes, cancer, and age-related diseases (36).

If we consider the clinical conditions of severe COVID-19 disease, there is evidence of recreating as well as GSH fullness (37).

Age is a major risk factor for disease and mortality in patients with COVID-19 (5). Compared to young mice, older mice have lower GSH in several organs. The severe decrease in glutathione in the lungs is considerable (38). Similarly, several studies have reported that in human samples, GSH concentrations decrease due to increasing age (39).

The severity of COVID-19 is also related to gender. Although the prevalence of infection is the same in both men and women, men with COVID-19, regardless of age, are more prone to worse outcomes and death (40).

According to several studies, GSH levels in men have decreased compared to those of women. Research on the effects of sex hormones on the production of free radicals and lipid peroxides has shown that red blood cell glutathione levels are lower in healthy men than in healthy women, and concluded that a decrease in GSH concentration is not a result of reduced production, but

probably is a result of the faster use against the increase of testosterone-induced oxidative stress (41).

For this reason, antioxidant therapies have beneficial effects on many diseases, characterized by inflammation and consequent impairment of redox homeostasis (42).

All of these studies show that the expression of antioxidants, including glutathione, is higher under oxidative stress, and when ROS is produced in large quantities.

However, high levels of ROS and faster use of glutathione against oxidative stress, due to reduced glutathione compared to high ROS, and cell deprivation of glutathione, will damage lipids and proteins, and even genes, and ultimately, cell destruction.

In the performed study, the expression of glutathione peroxidase and glutathione reductase genes, which are factors of increased ROS and oxidative stress, was evaluated in two groups of patients with severe and mild COVID-19 symptoms. The results showed an increase in the expression of glutathione peroxidase and glutathione reductase in the group of patients with severe symptoms, compared to patients with mild symptoms. However, in patients with severe symptoms, more cell damage was seen due to the high ROS and rapid consumption of glutathione.

CONCLUSION

The present study and similar studies show that low GSH can be a major cause of hyperinflammatory reactions in COVID-19 patients with severe symptoms. Signs and research projects show that increasing GSH or glutathione therapy can reduce the number of patients with severe symptoms. Thus, clinical and molecular studies in patients with COVID-19 may be the starting point for finding new diagnostic and therapeutic solutions and discovering such possibilities.

Disclosure statement

The authors have no conflict of interest.

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