IMPORTANT BIOMARKERS THAT PLAY A ROLE IN THE CHRONIC OBSTRUCTIVE PULMONARY DISEASE PROCESS

VAŽNI BIOMARKERI OD ZNAČAJA ZA PROCES HRONIČNE OPSTRUKTIVNE BOLESTI PLUĆA

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Summary

Background: Chronic obstructive pulmonary disease (COPD) that includes multiple mechanisms such as inflammation, infection, smoking, hypoxia, and lack of antioxidant response can cause oxidative stress. In our study, we aimed to determine the changes in some oxidative stress [malondialdehyde and glutathione] and some cellular immunity markers (neopterin and TGF-β) in patients diagnosed with COPD and determine the damage to the organism.

Methods: While the high-performance liquid chromatography (HPLC) method (Immuchrom kit, Germany) was utilized to determine MDA, GSH and NP levels, the ELISA method was used for TGF-β levels.

Results: All obtained data regarding each parameter were compared with both COPD and healthy individuals and between parameters. There was a statistically significant difference between the control group of healthy subjects and COPD group in all parameters (p<0.05). A negative and correlation between oxidant MDA and antioxidant GSH parameters was determined (p=-0.394).

Conclusions: As a result, it was seen that oxidative balance changed in the patient group and cellular immunity increased. When the obtained data and literature are taken into account, these changes occurring in oxidative balance and cellular immunity are of importance in determining the development in the pathogenesis of COPD, treatment options and their risks for heart disease in advance.

Keywords: chronic obstructive pulmonary disease, oxidative stress, cellular immunity

Kratak sadržaj

Uvod: Hronična opstruktivna bolest pluća (HOBP) koja uključuje više mehanizama kao što su inflamacija, infekcija, pušenje, hipoksija i odsustvo antioksidantnog odgovora može izazvati oksidativni stres. U našoj studiji, cilj je bio da se utvrde promene u nekim markerima oksidativnog stresa (malondialdehid i glutatoni) i čelijskog imuniteta (neopterin i TGF-β) kod pacijenata sa dijagnostom HOBP i procene oštećenja organizma.

Metode: Dok je za određivanje nivoa MDA, GSH i NP koristićena metoda HPLC (Immuchrom kit, Nemačka), za nivo TGF-β upotrebjen je metod ELISA.

Rezultati: Svi dobijeni podaci vezani za svaki parametar su upoređeni kod oboljelih i zdravih pojedinaca i između parametara. Pronađena je statistički značajna razlika između kontrolne grupe zdravih osoba i grupe sa HOBP za sve parametre (p<0,05). Negativna i jaka korelacija utvrđena je između oksidativnog parametra MDA i antioksidantnog parametra GSH (p=-0,394).

Zaključak: Kao rezultat, uočeno je da je u grupi oboljelih izmjenjen oksidativna ravnoteža i da je pojačan čelijski imunitet. Kad se uzmu u obzir dobijeni podaci i literatura, ove promene koje nastaju u oksidativnoj ravnoteži i čelijskom imunitetu od značaja su za određivanje razvoja u patogenezi HOBP, opcija lečenja i budućih rizika od srčanih bolesti.

Ključne reči: hronična opstruktivna bolest pluća, oksidativni stres, čelijski imunitet
**Introduction**

Chronic obstructive pulmonary disease (COPD) is a multifactorial condition which is the third leading cause of mortality after the cardiovascular diseases and affects millions of people around the world (1–4). COPD has a chronic nature with genetic predisposition characterized by progressive and irreversible airflow restriction occurring as a result of anti-oxidant and anti-protease systems impaired by smoking or several occupational-environmental factors (1, 5, 6). It produces a clinical course affecting severity and prognosis of the disease by features starting with the progressive airway obstruction and pulmonary involvement and then continuing with extra-pulmonary involvement. It is of importance, however, that it may be prevented by early treatment and it is a treatable condition (1, 6). Additionally, data from several studies show that one of the leading causes of death in COPD is ischemic heart disease (7, 8). Large-sized studies indicate that the COPD patients are at 2 to 3 times higher risk of cardiovascular mortality (8–10). Atherogenesis and the cardiovascular disease process are accelerated in patients with COPD. Key mechanisms in the pathogenesis of COPD include oxidative stress, decreased level of anti-oxidant protection, pulmonary and systemic inflammation, and endothelial dysfunction (4).

Oxidative stress has been defined as increased level of oxidants or decreased level of anti-oxidants. The oxidants are electron-acceptor molecules in the biological systems with the lungs being the most commonly involved (11). Several studies suggest that many mechanisms lead to oxidative stress. Especially, inflammation, several infections, hypoxia, exposure to tobacco smoke, decreased level of anti-oxidants may lead to oxidative stress (12, 13). Being an indirect marker that can detect the degree of oxidative damage in the systemic circulation and being a marker of oxidative stress, malondialdehyde (MDA) is formed as the result of oxidation of polyunsaturated fatty acids in the membrane structures impacted by free radicals (14).

Meanwhile, MDA is an end-product of lipid per-oxidation and is used as a marker of tissue reaction chain rate and in detecting reactive oxygen species (ROS). Plasma concentration of MDA is due to oxidative destruction of the lipid-peroxides (15). Glutathione is present in the cells in oxidized (GSSG) and reduced (GSH) forms. Its anti-oxidant effect occurs during the cycle between these two forms. Glutathione is one of the anti-oxidant substances protecting the cells from the destructive effects of free oxygen species forming during metabolic processes. Oxidative stress in the organism may be increased by many factors including environmental, metabolic, and individual ones and thus the formation of free radicals may increase. Thus, the level of GSH protecting cells from the destructive effects of these free radicals also varies. GSH level decreases and on the other hand the level of GSSG increases with increasing oxidative stress in the cell (16, 17).

Neopterin (NP) is one of the markers that may be used to detect activation of the cellular immune system (18). NP is an important marker for the coronary heart diseases and has been defined as a risk factor for acute respiratory infections in the patients with COPD (19). It is also a useful marker of oxidative stress, with its level responding to immunological stimulation rapidly. It may be detected more rapidly than erythrocyte sedimentation rate or soluble cytokine receptor concentration especially in the patients with inflammatory conditions (18). According to several studies, a strong correlation exists between inflammation and the extracellular matrix modulating cytokines (20–22). On the other hand, transforming growth factor-beta (TGF-β) plays an important role in re-inducing the airway in such chronic pulmonary diseases as COPD, asthma, and idiopathic pulmonary fibrosis (21). It is important for the airway remodeling. It may act as a regulatory key in many cellular and molecular processes (22, 23). Furthermore, TGF-β is an important polypeptide regulated by autocrine intracellular processes (23). It also has important effects including stimulation of chemotaxis of the inflammatory cells in wound healing (24). Increased expression of TGF-β1 has been reported in some cardiovascular diseases. Thus, TGF-β1 expression may contribute to progression of some cardiac conditions (25).

The present study aimed to detect damage in the organism by determining the changes in some markers of oxidative stress, anti-oxidant levels, and cellular immunity in patients with the diagnosis of COPD and emphasize its effects on mortality and morbidity of cardiac conditions.

**Material and Methods**

Our study includes patients diagnosed with COPD and being treated, with their stage of disease determined, in Corum Chest Diseases Hospital. According to the power analysis conducted before the study, 80 patients diagnosed with COPD and 30 healthy subjects (control group) were included in the study.

**Storage of samples**

Serum samples were obtained from the related center under the supervision of the project manager, protected from light and stored in a freezer at -86 °C until the work was done.
Determination of malondialdehyde (MDA) and glutathione (GSH) levels

Immuchrom kit in an HPLC system was utilized in the determination of MDA and GSH levels (HPLC Agilent Technologies 1200 series, USA; kit: Immuchrom GmbH, Germany) (Table I).

Neopterin levels

Neopterin levels were determined using the HPLC method (High Performance Liquid Chromatography) used by Avci (2008) and Cakir (2010) (18, 26) (Table I).

Transforming growth factor-beta (TGF-β) levels

In the determination of TGF-β levels in serums, high-sensitivity TGF-β ELISA (Bendermed System Diagnostics, eBioscience, Austria, kit No. BMS249 / 4) kit was used (measurement wavelength: 450 nm, reference wavelength: 620 nm).

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software (SPSS Inc., Chicago, IL). All measurements were taken in triplicates. The data were expressed as mean ± SD. For assessing the normality of distribution and homogeneity of variances of the data, Shapiro-Wilk were used. According to Huck (27), the values of skewness and kurtosis must change between -1 and +1 in order for the data to show normal distribution. Accordingly, values obtained in this study have emerged that allow the condition of normality (Table II).

When all data were appropriate, parametric independent sample T test was used. To determine

| Working condition | System Parameters | | | |
|-------------------|-------------------|--|---|---|---|
| HPLC Model        | Agilent Technologies 1200 Series | | | | |
| Sensor            | Flouresans Sensor | | | | |
| Mobile Phase      | -                 | -| -| 6.4 pH 0.015 mol/L phosphate buffer |
| Column material   | Bischoff Prontosil Eurobond, 5 μm; 125 mm × 4 mm | MZ Inertsil ODS, 5 μm; 125 mm × 4 mm | Analytical Column 4.6 × 250 mm, Allsphere ODS-2, C_{18} 5 μm reverse-phase |
|                   |                   | | | | |
| Wavelength        | 515 nm excitation and 553 nm emission | 385 nm excitation and 515 nm emission | 553 nm excitation and 438 nm emission |
| Flow Rate         | 0.8 to 1.0 mL/minute | 0.75 to 1.0 mL/minute | 0.8 mL/minute |
| Sample Volume     | 20 μL | | | |
| Operation time    | 4 minute | | | 20 minute |
| Temperature       | 30 °C | | | |
| Discrimination    | - | | | | |

| Table I | The working conditions and system parameters of the HPLC system used in the MDA, GSH and neopterin studies. |

| Working condition | System Parameters | | | |
|-------------------|-------------------|--|---|---|---|
| HPLC Model        | Agilent Technologies 1200 Series | | | | |
| Sensor            | Flouresans Sensor | | | | |
| Mobile Phase      | -                 | -| -| 6.4 pH 0.015 mol/L phosphate buffer |
| Column material   | Bischoff Prontosil Eurobond, 5 μm; 125 mm × 4 mm | MZ Inertsil ODS, 5 μm; 125 mm × 4 mm | Analytical Column 4.6 × 250 mm, Allsphere ODS-2, C_{18} 5 μm reverse-phase |
|                   |                   | | | | |
| Wavelength        | 515 nm excitation and 553 nm emission | 385 nm excitation and 515 nm emission | 553 nm excitation and 438 nm emission |
| Flow Rate         | 0.8 to 1.0 mL/minute | 0.75 to 1.0 mL/minute | 0.8 mL/minute |
| Sample Volume     | 20 μL | | | |
| Operation time    | 4 minute | | | 20 minute |
| Temperature       | 30 °C | | | |
| Discrimination    | - | | | | |

| Table II | Skewness and kurtosis values of all parameters. |

|          | Skewness | Skewness STD Error | R^1 | Kurtosis | Kurtosis STD Error | R^2 |
|----------|----------|--------------------|-----|----------|--------------------|-----|
| MDA      | 0.753    | 0.23               | 0.523 | -0.251   | 0.457              | -0.708 |
| GSH      | 0.379    | 0.23               | 0.149 | -0.439   | 0.457              | -0.896 |
| NP       | -0.956   | 0.23               | -1.186 | -0.944   | 0.457              | -1.401 |
| TGFβ     | -0.972   | 0.23               | -1.202 | -0.876   | 0.457              | -1.333 |

R^1 = Skewness - Skewness STD Error,
R^2 = Kurtosis - Kurtosis STD Error, R^2 ≤ | Kurtosis | = normal distribution
the association between MDA and GSH tested parameters, Pearson correlation coefficient was used. The critical significance level for the statistical tests performed was set to be 0.05.

Ethics

This study was approved by the local ethical committee (Protocol 01-01/2014).

Results

The serums of 80 COPD patients and of 30 healthy individuals in the control group were included in the study. The mean age for COPD patients enrolled in our study was 54±7, while the mean age for the control group was detected to be 48±4. The gender and age data of all patients are presented in Table III.

When, as markers of oxidative stress, oxidant MDA and antioxidant GSH data were evaluated, statistically significant changes were observed compared to the healthy control group. While MDA level in the control group was 1.38 ± 0.16 mmol/L, it was determined to be 1.93 ± 0.48 mmol/L in COPD patients. When it comes to the antioxidant indicator GSH, control group data determined to be 394.77 ± 30.65 mmol/L was seen to decrease in COPD group to 275.21 ± 41.19 mmol/L. Statistically significant difference between the groups was observed in both parameters (p<0.05) (Table IV, Figure 1). In addition, since MDA and GSH parameters of the data were parametric, negative and strong correlations between the two parameters were determined following Pearson correlation test (p=-0.394).

In our study, when the immunity markers NP and TGF-β data were studied, both indicators in COPD patients were detected to have increased compared to healthy subjects making up the control group (Table IV, Figure 2). In addition, statistically significant differences were found between the groups in both parameters (p<0.05).

Discussion

Chronic obstructive pulmonary disease (COPD) is a chronic condition which develops with inflammation caused in response to harmful gases and particles and which forms progressive and irreversible airflow limitation (2, 28). Since oxidative stress plays a central role in the pathogenesis of COPD, it must always be determined and in addition to smoking and childhood exposure to such harmful gas particles may also lead to the development of COPD (28, 29). Following the literature search results, the investigations of such parameters as antioxidant status, total oxidative status, various interleukins, C-reactive protein (CRP), tumor necrosis factor α were generally given weight in COPD patients and the importance of

Table III Demographic data of groups, both healthy-control and patients with COPD.

| Gender (Male/Female) | Control (n=30) | COPD (n=80) |
|----------------------|---------------|-------------|
|                      | 17/13         | 35–65       |
|                      | 48/32         | 45–73       |

Table IV Values of oxidative stress markers (MDA and GSH) and immune markers (NP and TGF-β) of patients with COPD and control group and their 95% confidence intervals.

| Parameters | Groups | n   | #Mean±SD | 95 % CI |
|------------|--------|-----|----------|---------|
|            |        |     |          | Min-Max |
| MDA, μmol/L* | Control | 30  | 1.38 ± 0.16 | 1.31–1.44 |
|            | COPD   | 80  | 1.93 ± 0.48 | 1.82–2.03 |
| GSH, μmol/L* | Control | 30  | 394.77 ± 30.65 | 383.32–406.21 |
|            | COPD   | 80  | 275.21 ± 41.19 | 266.04–284.57 |
| NP, μmol/L* | Control | 30  | 16.28 ± 1.06 | 15.88–16.68 |
|            | COPD   | 80  | 38.15 ± 1.86 | 37.73–38.56 |
| TGF-beta, pg/mL* | Control | 30  | 4.28 ± 0.40 | 4.13–4.43 |
|            | COPD   | 80  | 8.74 ± 0.41 | 8.64–8.83 |

MDA: Malondialdehyde, GSH: Glutathione, NP: Neopterin; TGF-beta: Transforming growth factor-beta; CI: Confidence interval for mean; #Each of groups is mean value and standard deviation. *p<0.005; statistically significant differences were found between the groups.
better nutrition was emphasized (3, 19, 28, 30). In our study, investigating such parameters as oxidant (MDA), antioxidant (GSH) and cellular immunity markers (neopterin) and cytokine levels (TGF-β) of the patients who received COPD treatment, the data obtained were compared with a healthy control group and an assessment was made to decrease mortality and morbidity in terms of some diseases.

According to our study, MDA levels as a marker of lipid peroxidation in the COPD patients were determined to have increased compared to the healthy control group. Similarly, in studies conducted by many researchers related to oxidative stress, it was also seen that MDA level increased among patients compared to the control group (28, 31, 32). In our study, GSH, an important antioxidant and protector of cells from the destructive effects of free oxygen radicals, was seen to have decreased in COPD patients compared with the healthy control group.

Different from our study, Drost et al. (33) found that GSH levels increased in the patient group compared to the control group. However, what was different in their study was that they studied the GSH parameter in bronchoalveolar lavage fluid. The results of studies reveal that the bronchoalveolar lavage technique is not suitable for diagnosis and is reported to have very low sensitivity for oxidative stress studies. In a study by Maury et al. (34, 35) in which they investigated the heterogeneity of systemic oxidative stress profiles in COPD patients, it was reported that GSH levels were quite low in COPD patients compared to the control group (36). Liu et al. (37) also reported in
a study in which they investigated the effect of cigarette smoke extracts on skeletal muscle cell aging by causing oxidative stress that they particularly observed a significant decrease in SOD and GSH activities.

When the formation mechanism of the important and precise marker of cellular immunity, neopterin, is examined, it is seen that the stimulated lymphocytes produce IFN-gamma, which activates macrophages and NP is synthesized from activated macrophages (38). NP both regulates the intracellular redox state and allows translocation of subunits of nuclear factor kB to the nucleus (39, 40). NP is regulated by a pro-inflammatory gene, so the clinical use of NP as an early inflammatory marker is important. Rising concentrations of NP are seen in patients with condensed monocyte/macrophage activity (41). Monocyte/macrophage increase is often encountered in people with heart diseases. Therefore, a relationship between NP and atherosclerosis was determined in the studies done.

Also, especially NP concentrations reflect the oxidative stress caused by the activation of the immune system (42–45). In the studies we have done so far, NP levels in COPD patients were found to increase compared with healthy individuals. Similarly, in a study by Rbyka et al. (46) in which they investigated the link between COPD and depression, to further investigate the inflammatory status of the patient, they assessed the cellular immune activation by measuring serum neopterin levels. As a result, they found a statistically significant increase of neopterin levels in patients with COPD compared to the control group. TGF-β is one of the most important growth factors in the pathogenesis of COPD (47). When these studies are investigated, it can be seen that the inflammatory cytokine TGF-β level is higher compared to healthy individuals (48, 49).

In a study by Zhang et al. (49) in which they identified several markers in patients with COPD, it was also seen that TGF-β level increased significantly compared to the control group. In our study, TGF-β levels of COPD patients were determined to have increased approximately two times as compared to healthy subjects. When the present studies were examined, we encountered quite a few studies that investigated serum neopterin and TGF-beta levels. It was also seen that there are not enough studies conducted to determine the role of oxidative stress markers. The data we obtained and literature search show that oxidant balance varies in this group of patients and that cellular immunity increases. When all the parameters are considered together, by referring to the burden on the development of COPD and the organism, we could determine that it created significant changes in several parameters.

In conclusion, it was also seen that the oxidant-antioxidant balance varies in COPD patients and that the levels of cellular immunity are directly or indirectly associated. It is also thought that the molecular changes in the levels of the oxidants and immunological agents caused by COPD might be risk factors for the development of cardiovascular disease in these patients. At the same time, our study is believed to shed light to the future studies to be conducted to determine both oxidative conditions and immunity in this patient group. Also, the effects of treatment protocols to be applied on oxidative stress antioxidants and on immune mediators are also believed to play a major role in the prognosis of the disease in these patients.

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Conflict of interest statement

The authors declare that they have no conflicts of interest for this work.
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