Bakteriyel sepsiste intrasellüler ve ekstrasellüler tiyol/disülfit homeostazisi

Intracellular and extracellular thiol/disulfide homeostasis in bacterial sepsis

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ÖZET

Amaç: Bakteriyel sepsis (BS) patogenezinde birçok mediatör ve biyobelirteç rol oynamakta olup tam olarak aydınlatılamamıştır. Çalışmanın amacı oksidatif stresin BS patogenezine katkısını değerlendirmek için BS'de hücre içi okside glutatyon (GSSG)/indirgenmiş glutatyon (GSH) ve serum tiyol/disülfid (hücre dışı) homeostazis testlerini analiz etmektir.

Gereç ve yöntem: Bu çalışmaya 49 hasta ve 43 sağlıklı gönüllü olmak üzere toplam 92 kişi dahil edildi. Sepsis ve septik şok için üçüncü uluslararası konsensüs tanımlarında tanımlanan BS kriterlerinin yüksek olasılık ve pozitif kan kültürü olan olgular çalışmaya dahil edildi. Kan örnekleri, hücre içi ve hücre dışı tiyol/disülfid homeostazis testleri için kullanıldı.

Bulgular: BS ve kontrol grubu karşılaştırıldığında, BS grubunda GSH, total glutatyon (GSH+GSSG), disülfid, İndeks-3, nativ tiyol ve toplam tiyol düzeyleri kontrol grubuna göre anlamlı derecede düşüktü (İndeks-3 için $p<0,001$ ve disülfid için $p<0.05$). GSSG düzeyleri açısından iki grup arasında anlamlı fark saptanmadı. BS grubunda yaş, CRP, PCT, WBC ve RDW, MCV, N/L ve Index-2 düzeyleri kontrol grubuna göre anlamlı derecede yüksekti ($p<0.05$).

Sonuçlar: BS'de total tiyol ve nativ tiyol düzeyleri ile GSH ve total glutatyon düzeyleri azaldı. Bozulmuş hücre içi ve hücre dışı tiyol/disülfid homeostazisinin oksidatif stresi artırarak BS patogenezi üzerinde etkileri olabilir.

Anahtar sözcükler: sepsis, redükteglutatyon, tiyoller, oksidatif stres

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Kısaltma listesi

GSSG: oksitlenmiş glutatyon

GSH: redüklenmiş glutatyon

GSH + GSSG: Total glutatyon

PCT: prokalsitonin

BS: bakteriyel sepsis

Intracellular and extracellular thiol/disulfide homeostasis in bacterial sepsis

Oxidative stress in bacterial sepsis

ABSTRACT

Aim: Many mediators and biomarkers play a role in the pathogenesis of bacterial sepsis (BS) which is still not fully illimunated. The aim was to analyze intracellular oxidised glutathione (GSSG)/reduced glutathione (GSH) and serum thiol/disulfide (extracellular) homeostasis tests in BS to evaluate the contribution of oxidative stress to pathogenesis.

Material and Methods: A total of 92 individuals, including 49 patients and 43 healthy volunteers, were included in the present study. The cases who had high probability of BS criteria defined by the third international consensus definitions for sepsis and septic shock and positive blood culture were included in the study. The blood samples were used for intracellular and extracellular thiol/disulfide homeostasis tests.

Results: When BS and control group were compared, GSH, total glutathione (GSH+GSSG), disulfide, Index-3, native thiol and total thiol levels were significantly lower in BS group compared to control group ($p<0.001$, for Index-3 and disulfide $p<0.05$). No significant differences were detected between the two groups in terms of GSSG levels. The age, CRP, PCT, WBC and RDW, MCV, N/L and Index-2 levels were significantly higher in BS group than in control group ($p<0.05$).

Conclusions: Total thiol and native thiol levels and GSH and total glutathione levels decreased in BS. Disrupted intracellular and extracellular thiol/disulfide homeostasis might have effects on BS pathogenesis by increasing oxidative stress.

Keywords: Sepsis, reduced glutathione, thiols, oxidative stress.

Abbreviation list:

GSSG: oxidised glutathione

GSH: reduced glutathione

GSH + GSSG: Total glutathione

PCT: procalcitonin

BS: bacterial sepsis

INTRODUCTION

Sepsis is one of the major causes of mortality and morbidity all over the world, and causes oxidative damage and multiple organ disorders. Sepsis is a common cause of death in both emergency departments and in intensive care units. Cell damage that is associated with oxidative stress is effective in the pathophysiology of sepsis. The damages at microcirculation level cause multiple organ failure (1-3).

Sepsis mortality rates varied between 18% and 40% in recent years (4-6). The highest mortality rates are associated with sepsis that is caused by infections of organisms that play roles in sepsis, such as bacteria and fungi. The other cause of the sepsis is virus (7, 8). Bacteria (*Staphylococcus aureus*) and fungi (*Candida* species) are generally detected in the culture of the sepsis patient (9). The response of the host immune system and the pathogen type have important role in sepsis severity (10). One of these is the altered of oxidation/ antioxidation balance in favor of oxidation (11). Different parameters have been examined in this respect in recent years, and

one of them is the determination of the level of glutathione in erythrocytes (4).

Glutathione is an important antioxidant, and is also a peptide that contains low-molecular-weight thiole. It has oxidised (GSSG) and reduced (GSH) forms in the cell, and gives information about oxidation-antioxidant status. Glutathiones have role in many metabolic processes, such as neutralizing the free radicals and peroxides, conjugation of the toxic metabolites, cell signaling, and transferring amino acids into the cells. For this reason, GSH/GSSG homeostasis is very important, and is associated with many cases (12, 13).

Erythrocytes are nucleus-free cells carrying oxygen, and are exposed to oxidative stress more than other cells. Also, free oxygen radicals are produced continuously in erythrocytes with auto-oxidation. However, since there are no protein molecules in erythrocytes that can break down these radicals, these radicals are neutralized by glutathione, which is a powerful antioxidant. For this reason, oxidative stress is responsible for the deterioration of GSH/GSSG homeostasis in erythrocytes (12, 13). The increase in the oxidative stress in erythrocytes causes cell damage, reducing in number in tissues in meeting oxygen needs.

Serum dynamic thiol/disulfide homeostasis play noteworthy and vital roles in detoxification, protection from oxidants, apoptosis, signal transduction, organizing of enzymatic activity, and in the transcription factors and cellular signaling mechanisms. Thiols are considered as the main antioxidant buffers of the extra-cell area in the serum (14).

Intracellular GSH and GSSG levels can be analyzed more practical and cost-effectively with the novel developed method (13) of Alışık et al. The accuracy, precision, and reproducibility of the new method is similar with in existing spectrophotometric methods. In this study, the purpose was to compare the intracellular oxidised/reduced glutathione and dynamic thiol-disulfide levels in the serum to evaluate the contribution of oxidative stress to etiopathogenesis in bacterial sepsis.

MATERIAL AND METHODS

Patients/material

The present study was conducted in Ankara City Hospital between May and September 2020. The study protocol was approved by the institutional ethics committee with the number of E1-20-437. A total of 49 patients diagnosed with sepsis and a healthy control group with 43 people were involved in the study. Written and verbal informed consent was obtained from all patients.

Patients who met the sepsis criteria defined by Singer et al. were included in our study based on clinical and laboratory findings (15). These criteria are grouped under 4 headings: 1) to have at least 3 clinical signs of sepsis (presence of fever, tachycardia/ bradycardia, necessity for oxygen support, need for ventilation, hypotension, feeding difficulty, abdominal distension), 2) CRP level >1 mg/ dL 3) Change in at least 2 different serum parameters (WBC count, platelet count, etc.) in addition to CRP, 4) positive blood culture or clinical sepsis (when blood culture is negative).Those younger than 18 years, those with chronic diseases such as diabetes mellitus, chronic renal failure and rheumatological disease, oncological patients, pregnant women and those who had trauma were not included in the study.

Sodium hydroxide (NaOH), Trizma base, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent), hydrochloric acid (HCl), ethylenedinitrilotetraacetic acid (EDTA), sodium borohydride (NaBH4), L-glutathione reduced (GSH), L-glutathione oxidised (GSSG), 2-mercaptoethanol, methanol, dithiothreitol (DTT), trichloroacetic acid (TCA), hydrogen peroxide solution (H2O2) and sodium chloride (NaCl) were purchased from Sigma-Aldrich Inc. (Taufkirchen, Germany) and Merck Co. (Darmstadt, Germany). Ultrapure-grade chemicals and Type-1 reagent-grade deionized water were used.

The complete blood count (CBC), CRP, and procalcitonin (PCT) were measured in the blood samples drawn from the patients with sepsis at diagnosis. Biochemical analyses were performed using an Atellica CH Analyzer (Siemens Diagnostic Product Corporation, Los Angeles, CA); CBC testing was performed using XE-2100 (Sysmex Corporation, Kobe, Japan).

Blood samples were obtained from healthy control group and sepsis group for serum thiol-disulfide measurements in the early morning after 8 hours of fasting. Serum samples were centrifugated at 1500g for 15 min, and stored at -80°C until the time of analysis. The type and manufacturers of the specimen tubes were serum separator tubes, BD Vacutainer®, (SST II Advance, REF 367955, UK).

Methods

Blood samples were taken from patient for blood culture analysis and the blood samples were obtained simultaneously for intracellular and extracellular glutathione measurement. The blood samples were stored before any treatment was initiated. The blood samples of cases with positive blood cultures were separated to study thiol-disulfide /glutathione and other parameters. Measurements were not affected since blood samples were taken for blood culture and other parameters before treatment was initiated. Those whose blood samples were taken after the treatment was initiated were excluded from the study.

Whole blood samples taken from healthy control group and sepsis group placed into BD Vacutainer® Plus Plastic K_2EDTA tubes (REF 367842, UK). Whole blood samples were pretreated as described in the study by Alışık et al. (13).

Serum concentrations of hsCRP were measured by a Tinaquant CRP (Latex) high sensitive immuno-turbidimetric assay on the Atellica CH hsCRP analyzer (Siemens Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's instructions.

Serum levels of PCT were measured by sandwich immunoassay using Siemens ADVIA Centaur XPT system (Siemens Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer's instructions.

Oxidized (GSSG), reduced (GSH) and total glutathione levels (GSH+GSSG) in erythrocytes were determined in the whole blood with a colorimetric method developed by Alışık et al. (13). In summary, the samples were treated with TCA solution to reduce GSSG to GSH by using NaOH and $NaBH_4$. GSSG molecules reduced with NaOH by increasing pH.

$NaBH_4$ was used as reductant. After the reduction phase, HCl solution which eliminate the remnant $NaBH_4$ prevented extra-reduction of DTNB molecules and re-oxidation of GSH molecules. GSH levels were obtained based on the Ellman method that was modified by Hu (16), which was using 500 mM Tris solution (pH: 8.2). Before and after the reduction phase, GSH was measured. After multiplication with a dilution factor, GSSG amount was calculated after subtraction of the GSH from the total GSH (GSH + GSSG) result and was divided by two.

Extracellular thiol/disulfide homeostasis was analyzed with automated and spectrophotometric method developed by Erel and Neselioglu (14). The basis of the thiol/disulfide measurement method was described in the study of Kalkan et al. (17). Index-1 (disulfide/native thiol%), index-2 (disulfide/total thiol%) and index-3 (native thiol/total thiol %) were calculated.

Statistical analysis

The statistical analyses were performed using the IBM SPSS Statistics (Version 22) computer program (IBM, Armonk, NY, USA, 2011). The Kolmogorov-Smirnov test were used for determing of distribution of variables. Variables with Gaussian distribution were analyzed with parametric tests and variables with non-Gaussian distribution were analyzed with non-

parametric tests. The Student's t test or was used for the comparison of normally distributed data variables. The Mann-Whitney U test was analyzed for comparison of the non-normally distributed data variables.In the correlation analysis, Pearson correlation analysis was performed for the normally distributed data variables, and Spearman's correlation analysis was performed for the non-normally distributed data variables.Chi-square test was used for categorical variables for two group comparisons. A p value of <0.05 was considered statistically significant. G*Power software version 3.1.9.2 was used to estimate required sample size for this study. The study power was set at 90% and alpha value set at 0.05. Based on these, a minimum sample of 40 subjects is required.

RESULTS

The demographic data of the BS and control groups are shown in Table 1. There was no significant difference in terms of gender, GSSG, Index-1 and PLT between the sepsis and control groups (Table 1). The age, CRP, PCT, WBC and RDW, MCV, N/L and Index-2 values of sepsis group were significantly higher than control group (p< 0.05, Table 1). Albumin, hemoglobin, GSH, GSH+GSSG, Index-3, native thiol, disulfide and total thiol levels were significantly lower in the sepsis group (p< 0.05, Table 1). İntracellular and extra- cellular thiol/disulfide homeostasis of control and BS groups are shown in Fig.1.

The correlations between PCT and CRP levels and intracellular and extracellular thiol/disulfide homeostasis tests are shown in Table 2 and 3. The correlation graphs are given in Fig. 2. PCT was negatively correlated with GSH (r= -0.309, p<0.05) and native thiol (r= -0.390, p<0.05) in sepsis group (Table 3 and Fig.2.). CRP was negatively correlated with GSH (r= -0.423, p<0.05), native thiol (r= -0.437, p<0.05) and total thiol (r= -0.418, p<0.05) in sepsis group (Table 2 and Fig.2.). There was no correlation between CRP, PCT values and disulfide and GSSG levels in the groups (Table 2 and Table 3).

Table 1. Demographic characteristics of sepsis and control groups.
Tablo 1. Sepsis ve kontrol gruplarının demografik özellikleri.

Parameters	Control group (n=43)	Sepsis group (n=49)	p Value
Age, mean ± SD, range, years	58.4 ± 16.6, 20-87	68.2 ± 16.9, 18-93	0.006*
Gender, female/male	19/24	22/27	0.945
CRP, mean ± SD, g/L	0.004 ± 0.003	0.18 ± 0.04	<0.001*
PCT, mean ± SD, μg/L	0.03 ± 0.02	10.7 ± 9.6	<0.001*
WBC, mean ± SD, 10^9/L	7.5 ± 0.9	15.5 ± 7.2	<0.001*
MCV, mean ± SD, fL	85.4 ± 2.8	89.4 ± 6.8	0.048*
RDW, mean ± SD,%	13.3 ± 0.9	15.5 ± 1.5	<0.001*
N/L, mean ± SD	2.3 ± 0.7	18.8 ± 20.2	0.024*
PLT, mean ± SD ,10^9/L	213.8 ± 45.4	196.7 ± 113.3	0.693
Hemoglobin, mean ± SD, g/dL	15.4 ± 1.5	12.6 ± 1.7	<0.001*
Albumin, mean ± SD, g/L,	45.4 ± 1.7	32.2 ± 5.4	<0.001*
GSH, mean ± SD, μmol/L,	738.7 ± 91.8	619.5 ± 57.3	<0.001*
GSH + GSSG, mean ± SD, μmol/L	984.4 ± 157.1	830.9 ± 93.9	<0.001*
GSSG, mean ± SD, μmol/L	122.8 ± 70.3	105.8 ± 44.9	0.236
Native thiol, mean ± SD , μmol/L	317.9 ± 78.3	184.7 ± 69.3	<0.001*
Total thiol, mean ± SD , μmol/L	388.6 ± 86.7	246.5 ± 73.8	<0.001*
Disulfide, mean ± SD , μmol/L	73.4 ± 95.1	32.5 ± 9.9	0.005*
Index-1, mean ± SD	18.7 ± 9.8	20.6 ± 10.2	0.013*
Index-2, mean ± SD	13.8 ± 6.3	14.9 ± 6.7	0.026*
Index-3, mean ± SD	73.7 ± 9.1	71.9 ± 9.3	0.030*

p Values less than 0.05 were considered significant highlighted in asterisk.

Table 2. Correlations between CRP and other parameters.
Tablo 2. CRP ve diğer parametreler arasındaki korelasyonlar.

	Control group (*n*=43)		Sepsis group (*n*=49)	
Parameters	***r***	***p* value**	***r***	***p* value**
GSH + GSSG, μmol/L	-0.111	0.695	-0.158	0.301
GSH,μmol/L	0.018	0.948	-0.423	**0.004**
GSSG, μmol/L	-0.136	0.630	0.100	0.513
Native thiol, mmol/L	-0.231	0.407	-0.437	**0.003**
Total thiol, mmol/L	-0.158	0.573	-0.418	**0.005**
Disulfide, mmol/L	0.027	0.930	-0.025	0.870
Disulfide/native thiol X 100%	-0.091	0.747	0.182	0.256
Disulfide/total thiol X 100 %	0.025	0.931	0.155	0.320
Native thiol/total thiol X 100, %	-0.028	0.922	-0.183	0.247
PCT, μg/L	0.695	**0.003**	0.339	**0.021**

p Values less than 0.05 were considered significant highlighted in bold.

Table 3. Correlations between PCT and other parameters.
Tablo 3. PCT ve diğer parametreler arasındaki korelasyonlar.

	Control group (*n*=43)		Sepsis group (*n*=49)	
Parameters	***r***	***p* value**	***r***	***p* value**
GSH + GSSG, μmol/L	-0.109	0.700	-0.24	0.878
GSH,μmol/L	0.042	0.881	-0.309	**0.039**
GSSG, μmol/L	-0.110	0.695	0.187	0.219
Native thiol, mmol/L	-0.061	0.830	-0.390	**0.008**
Total thiol, mmol/L	-0.002	0.995	-0.280	0.063
Disulfide, mmol/L	-0.064	0.835	0.129	0.399
Disulfide/native thiol X 100%	-0.355	0.194	0.058	0.718
Disulfide/total thiol X 100 %	-0262	0.645	-0.128	0.415
Native thiol/total thiol X 100, %	0.262	0.345	0.079	0.618
CRP, g/L	0.695	**0.003**	0.339	**0.021**

p Values less than 0.05 were considered significant highlighted in bold.

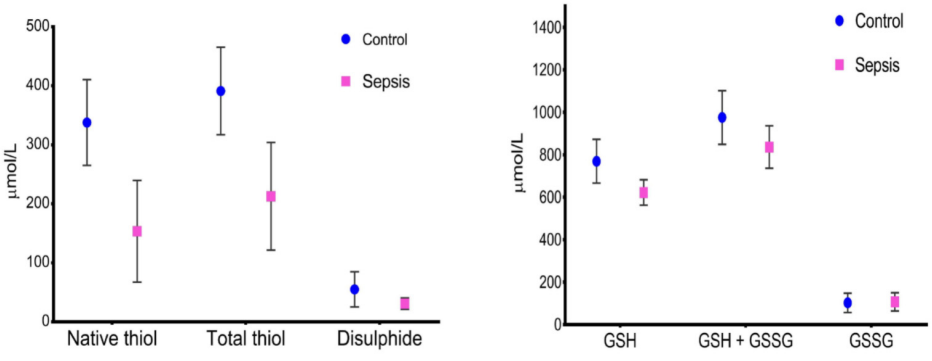

Fig.1. Comparison intracellular (A) and extracelullar (B) GSH and thiol/disulfide homeostasis levels of control and BS groups.

Şekil 1. Kontrol ve BS gruplarının hücre içi (A) ve hücre dışı (B) GSH ve tiyol/disülfid homeostaz düzeylerinin karşılaştırılması.

p values less than 0.001 were considered significant highlighted in asterisk.

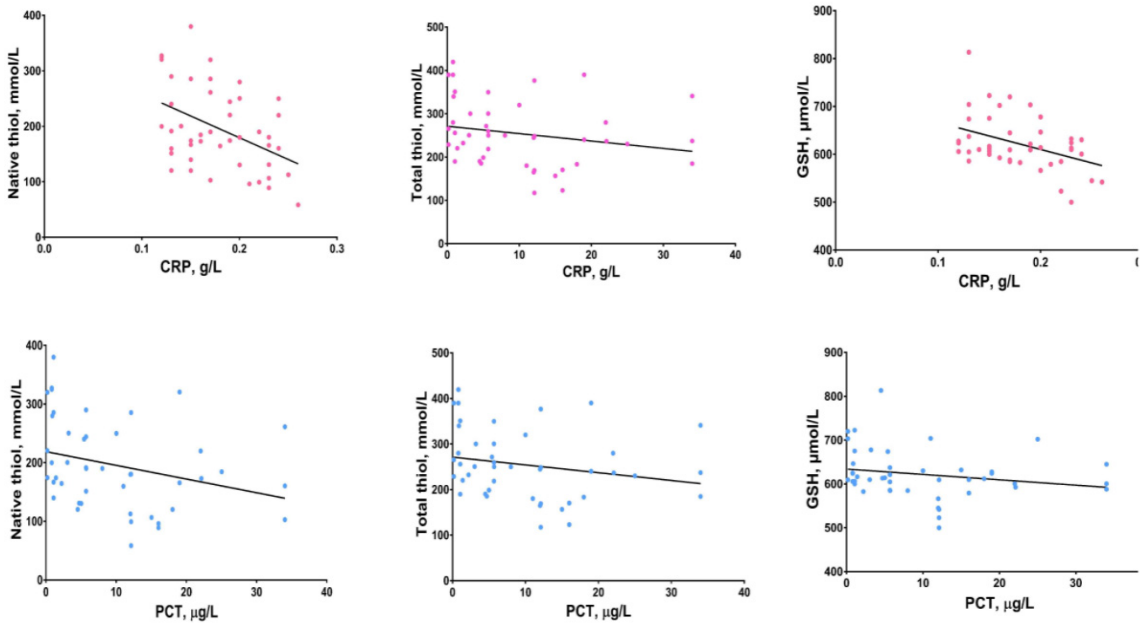

Fig.2. Correlation graphs of CRP with native thiol (A), CRP with total thiol (B) and CRP with GSH (C), PCT with native thiol (D), PCT with total thiol (E) and PCT with GSH (F).

Şekil 2. CRP ile nativ tiyol (A), CRP ile toplam tiyol (B) ve CRP ile GSH (C), nativ tiyol ile PCT (D), total tiyol ile PCT (E) ve GSH ile PCT (F) arasındaki korelasyon grafikleri.

DISCUSSION

GSH is the main intracellular thiol involved in cellular redox reactions and thioester formation. GSH has important roles in protecting erythrocyte and brain cells from oxidative stress, as well as in keeping the thiol redox status (12, 13, 18). Disruption in the oxidant/antioxidant balance, which is an important cause of cell damage, causes deterioration in cell functions. The change in the balance in favor of the oxidant plays roles in increasing morbidity and mortality by increasing cell damage.

Numerous studies were conducted on bacterial sepsis, its pathophysiology is not yet illuminated fully. Many compounds and biomarkers play a role in the etiopathogenesis of bacterial sepsis. Oxidative stress also plays important roles in its pathophysiology. Previous studies reported increased oxidative marker levels such as malondialdehyde, myeloperoxidase, superoxide dismutase/catalase ratio, and decreased antioxidant marker levels such as GSH, in sepsis (19, 20).

The decrease in the level of GSH, which represents a large proportion of antioxidants in the cell, causes damage by increasing oxidation. GSH levels were shown to be decreased in patients with BS in previous studies (8, 21,22). These studies focused on the oxidant or antioxidant aspect of the oxidant-antioxidant balance (20, 23). In these studies, the balance was not investigated in both ways and the methods used were time-consuming or expensive (chemiluminescence method, enzyme-linked immuno-sorbent assay etc.) (22, 23). In the present study, intracellular glutathione levels and extracellular thiols were measured with an automated and practical spectrophotometric method (13).

In this study, we also calculated GSSG and disulfide levels. In our study, GSH and GSH+GSSG were found to be low in bacterial sepsis with severe oxidative stress compared to the control group. The decrease in intracellular GSH levels in bacterial sepsis makes us consider the severity of the disease. It may be helpful to show the severity of the disease in the early period by measuring the level of intracellular GSH in bacterial sepsis. While GSH decreases, GSSG

is expected to increase, but in our study, while GSH decreased, GSSG levels were not changed significantly. These results may explain that there is no significant change in GSSG levels due to the advanced oxidation of GSSG. Advanced oxidation in BS may show us the severity of the disease. Some studies have reported cases of advanced oxidation (24, 25).

Dynamic thiol/ disulfide homeostasis play an important role in detoxification, protection from oxidants, signal transduction, regulation of enzymatic activity, cellular signaling mechanisms, and transcription factors. Thiols are known as essential antioxidant buffers because they interact dynamically with many physiological oxidants. Thiol protein groups are important antioxidants, which constitute 52.9% of total serum antioxidant capacity in healthy individuals (26).

The thiol-disulfide balance has been investigated in recent years in many studies by measuring serum level with a new method, which has been associated with the severity of oxidative stress (26-27). It was reported in the study that that native thiol, total thiol and disulfide levels were decreased in septic shock and bacterial sepsis patients, similar to our study results (28). In the current study, there was a significant decrease in the total thiol, native thiol and disulfide levels in BS compared to the control group. Our findings suggest that impaired thiol disulfide homeostasis plays a great role in the etiopathogenesis of BS. The disulfide levels increased with oxidative stress, which suggests that there may be advanced oxidation in bacterial sepsis. Erel and Neselioğlu reported that disulfide levels are lower in proliferative diseases such as diabetes mellitus and some malignancies. (14). Our findings were consistent with the literature (29).

CRP, PCT, WBC, MCV, RDW, and N/L, which are other nonspecific parameters, were found to be consistent with the literature data in our study. These parameters were nonspesific and significant in showing the inflammation degree, and were also high in bacterial sepsis (29). In additon, we found a significant negative correlation between GSH and CRP and PCT in line with the literature (21, 30).

The detection of intracellular and extracellular thiol levels with practical methods as antioxidants can make it easier for us to monitor the effectiveness of treatments. Comprehensive studies are needed on this issue.

The limitations of our study are the small sample size in the study population. Associated conditions that may cause oxidative stress may affect thiol levels.In the patients with unknown etiology of BS, there may be conditions affecting the oxidative stress. Does the giving thiol sources as treatment contribute to the reduction of oxidative stress in patients with BS? Further studies are needed to find answers to these questions.

CONCLUSION

Our findings provided evidence to suggest the disrupted oxidant/antioxidant balance based on decreased intracellular and extracellular thiol/disulfide in patients with BS. Disruption of the thiol/disulfide homeostasis may have an impact on BS pathogenesis by increasing oxidative stress. Evaluating the GSH/GSSG and serum thiol/disulfide homeostasis levels at admission may provide better care for patients with BS.

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