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Thiol/Disulfide homeostasis in patients with rheumatoid arthritis

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Background. Oxidative stress may play an important role in rheumatoid arthritis (RA) etiopathogenesis. The thiol group is a very strong antioxidant. In this study, we aimed to investigate the presence of oxidative stress in patients with RA by evaluating thiol/disulfide homeostasis.

Material and methods. A total of 50 female RA patients and 50 healthy female controls were included in this study. Thiol and disulfide values were calculated utilizing novel methods.

Results. Native thiol ($p < 0.001$) and total thiol ($p < 0.001$) levels of RA patients were significantly lower compared to values in the control group. However, the disulfide ($p < 0.001$) levels of RA patients were strongly higher than in healthy individuals. A negative correlation was found between thiol and disease activity score-28 among the patients, whereas a positive correlation was found between disulfide and disease activity score-28 among the patients.

Conclusion. We found that the thiol–disulfide rate deteriorated in RA patients, with the proportion of disulfide increasing. There is a strong correlation between the decrease in thiol levels, increase in disulfide levels and the disease activity scores.

Keywords: Rheumatoid arthritis, thiol, disulfide, thiol/disulfide homeostasis, oxidative stress.

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease. Generally, small joints of the hands and feet and large joints such as the knee, shoulder, and elbow are affected in RA. Its pathogenesis has not yet been clarified. However, genetic predisposition, environmental factors, and cellular and molecular mechanisms are known as main factors [1]. RA leads to hypertrophy and inflammation in the synovial membranes of the diarthrodial joints. If RA is not treated, there can be severe deformation and destruction in joints leading to subluxation, that is joint contractures. Various organs and systems are extra-articularly involved, and rheumatoid vasculitis can affect the eyes, skin, lung, and heart [2, 3].

The genetic disposition rate in RA is 65%, and it is known that HLADR4 and HLADR1 alleles are important predisposition factors for the disease [4]. Moreover, it is believed that complex cellular interactions have roles in RA pathogenesis, affecting CD4+ T cells, fibroblasts, B cells, dendritic cells, and macrophages [5]. These changes, which lead to RA pathogenesis, cause tissue damage in joint cavities with RA and in synovial fluid filled with inflammatory cells due to the production of reactive oxygen species (ROS) [6]. This increases intra-articular pressure, capillary permeability, and thus ischemia and perfusion damage [7]. Tissue damage leads to the release of free oxygen and nitrogen compounds and free radicals. Abnormal vascular changes contribute to this oxidative stress cycle, increasing the metabolic rate in the synovial tissue [8].

Oxidant–antioxidant mechanisms have important roles within organisms and in homeostasis. The "thiol" is used for organic compounds containing sulfhydryl (-SH) [9]. Recently, it has been stated that thiol groups can be used as an oxidative stress marker [10]. Thiol/disulfide measurements can be used to evaluate the free radical status of an organism both in physiological and pathological conditions [11]. Erel and Neselioglu developed an automated method that can separately measure thiol/disulfide groups, which change significantly in various diseases [12].

In this study, we aimed to investigate the presence of oxidative stress in patients with RA by evaluating thiol/disulfide homeostasis. In addition,

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we investigated whether or not the thiol/disulfide balance may be related to the disease activity score.

MATERIAL AND METHODS

STUDY DESIGN

We included 50 female patients (age- and body mass index (BMI)-matched) in the study. The RA patients were admitted to Malatya Education and Research Hospital Rheumatology outpatient clinic and were selected based on the diagnostic criteria of the American College of Rheumatology/ European League Against Rheumatism (ACR/ EULAR) 2010 [13]. These individuals who had mild pain such as myalgia and did not have any rheumatological or other diseases as a result of their physical examinations and laboratory tests were admitted to the internal medicine outpatient policlinic in our hospital as a healthy control group. Hemograms, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), visual activity score, disease activity score-28 (DAS-28), and basic clinical characteristics of the patients and control subjects were recorded.

We excluded patients with comorbid diseases (hypertension, diabetes mellitus, cancer, neurological and psychiatric diseases, serious systemic infections, acute or chronic organ failure, and genetic disorders). We recorded age, gender, disease duration, frequency of exacerbations, drug use, as well as the signs and symptoms related to the disease using a face-to-face interview technique. Individuals who were routinely treated with any type of medicine were not included in the control group. Patients who were using drugs other than a non-steroidal anti-inflammatory drug, disease-modifying anti-rheumatic drug (DMARD), or tumor necrosis factor alpha (TNF-α) blocker were not included in the RA group.

ETHICS APPROVAL

The procedures used in our study were in accordance with the revised form of the Helsinki Declaration 2013, and all participants signed an informed consent form. Our study protocol was approved by the Local Ethical Committee (Ankara Numune Education and Research Hospital).

BIOCHEMICAL ANALYSIS

Venous blood samples of both patients and controls were collected into a dry tube. Blood samples were centrifuged at 1000 g for 15 minutes without waiting. Serum samples were divided into different tubes and stored at -80ºC for analyses. Whole blood counting was performed using flow cytometry (Mindray BC-6800 Auto Hematology Analyzer, Shenzhen, China); CRP, rheumatoid factor (RF), liver, and kidney function tests were performed using the spectrophotometric method (Abbot-Architect c8000, Japan); and ESR was measured using the Westergren method (Berkhum SDM-100, Turkey). Analyses were performed in the biochemistry laboratory of the Malatya State Hospital.

THIOL/DISULFIDE MEASUREMENT

Serum native thiol (NT), total thiol (TT), and disulfide levels (μmol/L) were measured following the method developed by Erel and Neselioglu [12]. Disulfide/native thiol (DNT), disulfide/total thiol (DTT), and native thiol/total thiol (NTT) rates were calculated (%). Disulfide bonds were reduced to functional thiol groups, particularly in the presence of sodium borohydride. Then, the sodium borohydride was removed with the help of formaldehyde. Reduced and native thiol groups were measured after the 5,5'-dithiobis- (2-nitrobenzoic) acid (DTNB) reaction. Half of the difference between the total thiol and native thiol groups were calculated, and the amount of dynamic disulfide was measured.

STATISTICAL EVALUATION

Results were presented as mean ± SD and median (range). Statistical evaluation was conducted using the SPSS program (version 18, IBM, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to determine whether or not the groups were homogeneous. Data that were not normally distributed, such as CRP and ESR, were evaluated using the Mann–Whitney U test. Other findings that were normally distributed were evaluated using the Student t-test. Pearson's correlation test was used for correlation analysis. Statistical significance was accepted as $p \leq 0.05$.

RESULTS

DAS-28, disease duration, and drug treatment effects on RA patients can be seen in Table 1.

The NT (282.3 ± 48.6 *vs*. 339.8 ± 55.7 μmol/L, $p < 0.001$) and TT (319.4 ± 50.3 *vs*. 366.0 ± 49.6 μmol/L, $p < 0.001$) levels of RA patients were significantly lower than those of the controls (Figure 1). The NTT levels of RA patients were also significantly lower than those of the control group ($p < 0.001$) (Table 2). In contrast, the

disulfide (18.5 ± 7.2 *vs*. 13.1 ± 6.6 µmol/L, p < 0.001) levels of RA patients were strongly higher compared to controls (Figure 2). The DNT (p < 0.001) and DTT (p < 0.001) levels of RA patients were significantly higher compared to controls. Furthermore, the CRP and ESR values of RA patients were significantly higher compared to the CRP and ESR values of the control group (p < 0.001). Table 2 presents the biochemistry results of both RA and control group individuals.

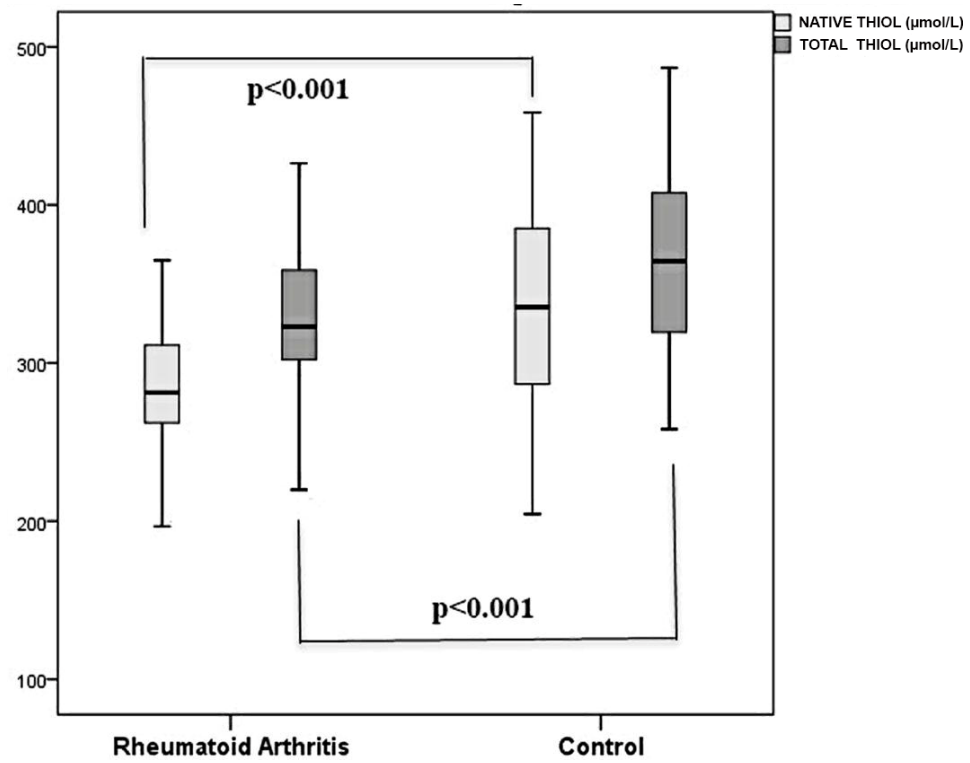

Figure 1. Native thiol and total thiol levels in rheumatoid arthritis.

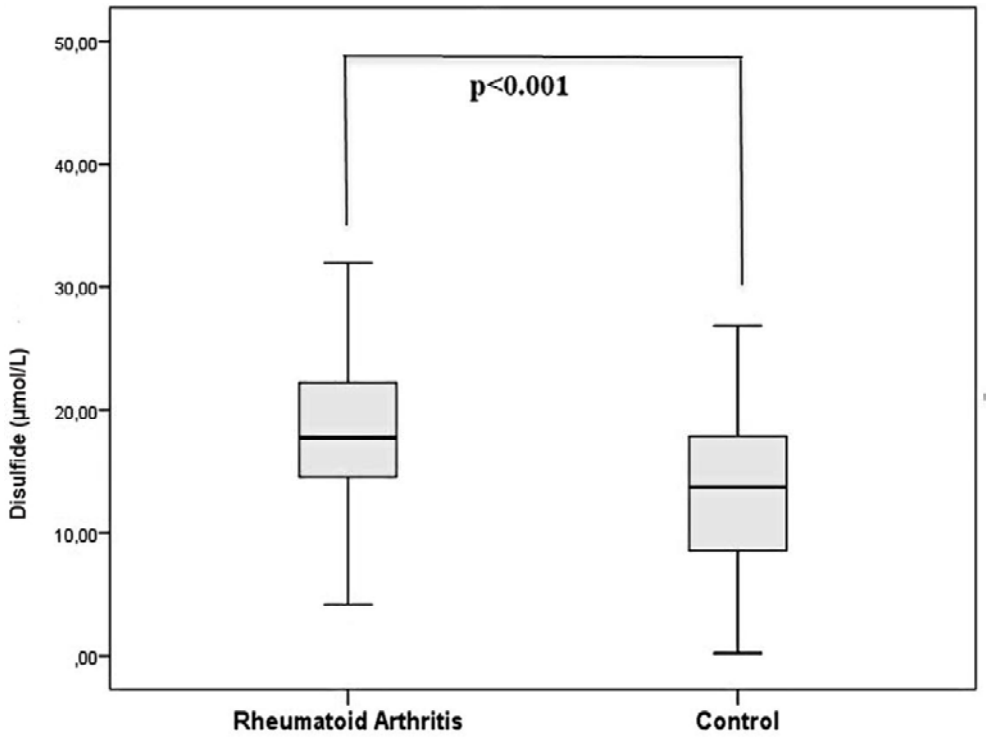

Figure 2. Disulfide levels in rheumatoid arthritis.

Table 1
The patient characteristics, disease activity and drug treatments

Parameters	**RA (50)**
Duration of disease (years)	5.5 (1.0-20.0)
DAS-28	3.4±1.1
RF (+/-)	38/12 (76.0%/24.0%)
Infliximab	2 (4.0%)
Etanercept	3 (6.0%)
Golimumab	2 (4.0%)
Adalimumab	2 (4.0%)
Hydroxychloroquine	30 (60.0%)
Salazopyrin	10 (20.0%)
Methotrexate	20 (40.0%)
Leflunomide	30 (60.0%)
Steroid	10 (20.0%)

Data are expressed as mean ± standard deviation, median (range), and n (%) where appropriate. RA, rheumatoid arthritis; BMI, body mass index; DAS-28, disease activity score-28; RF, rheumatoid factor.

Table 2
Comparisons of demographic features and native thiol, total thiol, and disulfide, measurements across groups

	RA patients (n = 50)	**Control (n = 50)**	**p value**
Age (years)	47.7 ± 10.3	45.8 ± 8.6	0.305[a]
BMI (kg/m^2)	26.6 ± 4.1	27.7 ± 5.5	0.258[a]
Native thiol/total thiol × 100	88.3 ± 4.7	92.4 ± 4.4	0.001[a]
Disulfide/native thiol × 100	6.7 ± 3.3	4.1 ± 2.7	0.001[a]
Disulfide/total thiol × 100	5.8 ± 2.3	3.7 ± 2.2	0.001[a]
ESR (mm/h)	27.0 (1.0-85.0)	13.5 (1.0-43.0)	0.001[b]
CRP (mg/dL)	0.7 (0.1-0.8)	0.3 (0.1-0.4)	0.001[b]
BUN (mg/dL)	24.3 ± 7.4	25.6 ± 6.7	0.343[a]
Creatinine (mg/dL)	0.66 ± 0.07	0.68 ± 0.08	0.222[a]
AST (IU/L)	18.9 ± 7.6	20.1 ± 8.7	0.467[a]
ALT (IU/L)	22.5 ± 12.5	20.0 ± 8.7	0.271[a]

[a]T test
[b]Mann–Whitney U test
Data are expressed as mean±standard deviation and median (min–max) where appropriate. P<0.05 indicates a significant difference. RA, rheumatoid arthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 3
Comparison of correlation analysis for native thiol, total thiol and disulfide levels, ratios, disease activity score, disease duration, and inflammation markers results among RA patients

	DAS-28		**Disease duration**		**CRP**		**ESR**	
	r	**p[a]**	**r**	**p[a]**	**r**	**p[a]**	**r**	**p[a]**
NT	**-0.462**	**0.001**	-0.194	0.176	-0.114	0.262	**-0.270**	**0.007**
TT	**-0.403**	**0.001**	-0.101	0.486	-0.112	0.268	**-0.259**	**0.010**
NT/TT	**-0.417**	**0.001**	**-0.355**	**0.011**	-0.057	0.575	**-0.198**	**0.049**
Disulfide	**0.358**	**0.001**	**0.304**	**0.032**	0.039	0.699	0.125	0.219
Disulfide/NT	**0.400**	**0.001**	**0.362**	**0.010**	0.039	0.701	0.201	0.046
Disulfide/TT	**0.414**	**0.001**	**0.355**	**0.011**	0.056	0.583	0.196	0.052
CRP	**0.421**	**0.001**	0.015	0.920			**0.449**	**0.001**
ESR	**0.553**	**0.001**	0.063	0.667	**0.449**	**0.001**		

[a]Pearson Correlation test. RA, rheumatoid arthritis; DAS-28, disease activity score-28; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; NT, native thiol; TT, total thiol.

According to the correlation analysis, there was a negative correlation between DAS-28 and NT, TT, and NTT, whereas the correlation between DAS-28 and disulfide, DNT, DTT, CRP, and ESR was positive. There was a negative correlation between disease duration and the NTT values. On the other hand, we detected a positive correlation between disease duration and the DNT and DTT

values. There was a negative correlation between ESR and NT, TT, and NTT. We detected a positive correlation between ESR and DNT values. However, there was no correlation between the thiol and disulfide levels and CRP values. In Table 3, a relationship can be seen between the thiol and disulfide levels and DAS-28, disease duration, CRP, and ESR levels.

DISCUSSION

The thiol values of the RA patients were lower than those of the controls, and disulfide levels were also higher compared to those of the controls. The thiol–disulfide rate deteriorated in RA patients, with the proportion of disulfide increasing. RA is a disease with inflammatory processes, such as an increase in cytokines including TNF-α and interleukin (IL)-6 [14]. Cytokine release increases oxidative stress and the formation of reactive oxygen radicals [15]. Inhibition of the cytokine release leads to a decrease in oxidative stress and ROS production [16, 17]. It has been reported that lipid peroxidation and oxidative stress increase in RA, which also leads to deterioration of the antioxidant defense systems [18]. Further, superoxide anion, hydrogen peroxide, and peroxynitrite radicals are increased in RA patients [19]. There is deterioration in the levels of antioxidants, such as superoxide dismutase, glutathione peroxidase, and catalase [20]. In addition, the total oxidative status and oxidative stability index were higher and the total antioxidant status was prominently lower in RA patients compared to controls [21]. Paraoxonase-1 enzyme activity was also lower in RA patients [22]. There was a strong correlation between ROS formation and DAS-28 in RA patients [23]. This greater oxidative stress increased the release of TNF-α in RA patients, increasing the severity of the disease [24].

Hypochlorous acid (HOCl) is a type of ROS that is released from neutrophils and macrophages [25]. Compounds that have roles in ROS removal, such as hypochlorous acid, peroxynitrite, and hydrogen peroxide, contain thiol groups (glutathione, methionine, and taurine) [26]. Therefore, the thiol/disulfide balance is very important. When this balance changes in favor of disulfide, ROS are produced [27]. Thiol groups that contain sulfhydryl, mostly in albumin and proteins and glutathione, are the most important thiol-containing groups [28]. The strong antioxidant effect of drugs containing thiol groups such as N-acetyl is well known. The measurement of thiol was previously performed using manual methods [29]. However, Erel *et al*. have recently developed an inexpensive, easy, reliable, and fully automated method that is also able to measure the thiol/disulfide balance [12]. Using this method, it was discovered that the balance of thiol/disulfide deteriorates in favor of disulfide in diseases characterized by increased oxidative stress, such as familial Mediterranean fever (FMF), hypertension, and diabetes mellitus [30-32]. In FMF patients, low paraoxanase-1 enzyme and increased macrophage migration inhibitory factor levels can be associated with deterioration of the thiol/disulfide balance [30]. Macrophage migration inhibitory factor levels also increase in RA patients [33]. Changes in antioxidant enzyme levels and increased antioxidant capacity in RA can be explained by the increased proportion of disulfide in the thiol/disulfide balance. Giustarini *et al*. determined that patients with RA have got lower thiol and higher disulfide level compared to healthy control [34]. Similarly to the results of Giustarini *et al*., it was observed that thiol levels were lower and disulfide levels were higher in RA patients compared to the controls in our study. There was a negative correlation between DAS-28 and thiol groups and disulfide levels. This change in the thiol/disulfide balance (in favor of disulfide) can increase the disease activity by leading to the release of TNF-α and other pro-inflammatory cytokines. Furthermore, the deterioration of the thiol/disulfide balance can have an impact on the etiology of RA.

It has been reported that TNF-α treatment increases paraoxonase (PON-1) levels and decreases oxidative stress [33]. Furthermore, tocilizumab treatment blocks IL-6 release and decreases ROS formation [35]. However, it has been found that sulfasalazine and methotrexate treatments decrease thiol groups and increase oxidative stress [36]. Very few of our patients were treated with anti-TNF, although thiol levels could be better in patients who were treated with anti-TNF. There are limited studies in RA patients about thiol-disulfide homeostasis but our study is the first in which thiol groups are examined in RA patients by using novel Erel's method. In this regard, more comprehensive studies should be performed with larger groups.

CONCLUSION

We found that the thiol–disulfide rate deteriorated in RA patients, with the proportion of disulfide increasing. There is a strong correlation

between the decrease in thiol levels, increase in disulfide levels, and the disease activity scores. Moreover, there is a strong correlation between the deterioration of the thiol–disulfide balance and the RA disease activity.

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Conflicts of interest: All authors report that there is no conflict of interest. In our study, no funding was received from any public or commercial institutions or for-profit organizations.

Introducere. *Stressul oxidativ poate juca un rol important in patogeneza artritei reumatoide (RA). Gruparea tiol este o grupare cu puternice proprietăți antioxidante. Scopul acestui studiu a fost de a evalua homeostazia tiol/disulfid la pacienții cu AR.*

Materiale şi metode. *50 de paciente cu RA şi 50 de martori sănătoşi au fost incluşi în studiu. Grupările tiol şi disulfidice au fost măsurate folosind metode noi de evaluare.*

Rezultate. *Nivelurile native de tiol (p;0.001) şi tiolul total la pacienții cu AR au fost semnificativ statistic mai mici comparativ cu valorile din grupul control. Totuşi nivelurile disulfidului la pacienții cu AR au fost mai mari decât la pacienții sănătoşi. S-a găsit o corelație negativă între nivelul tiolului şi scorul de activitate al bolii (DAS 28). S-a găsit o corelație pozitivă între nivelurile disulfidului şi scorul de activitate al bolii (DAS 28).*

Concluzii. *Raportul tiol/disulfid este deteriorat la pacienții cu AR, cu creşterea celui din urmă. S-a găsit o corelație între niveluruile tiolului şi a disulfidului şi scorul de activitate al bolii.*

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