Relationship of Cellular Oxidant and Antioxidant Status with Disease Activity in Patients with Rheumatoid Arthritis

Romatoid Artrit Hastalarında Hücresel Oksidan ve Antioksidan Statü ve Hastalık Aktivitesi İle İlişkisi

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Abstract

Objective: In recent years, increasing attention has been given to the role of reactive oxygen metabolites in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA). The aim of this study was to elucidate plasma and especially erythrocyte oxidant and antioxidant status in RA patients and to assess the relationship between disease activity scores (evaluated by Disease Activity Score (DAS) 28 as mild, moderate and severe) and antioxidant status.

Materials and Methods: Fasting blood samples were obtained from 50 RA patients and 26 control subjects. DAS28 was used to evaluate the activity. Plasma and erythrocyte levels of malondialdehyde (MDA), xanthine oxidase (XO) and superoxide dismutase (SOD) were investigated in both groups.

Results: The plasma levels of MDA and XO and erythrocyte levels of MDA and SOD were significantly higher in RA patients than in control subjects. Although increases in plasma MDA levels in mild and moderate and erythrocyte MDA levels in mild activity groups of RA were not significant ($p>0.05$), plasma MDA levels in severe ($p=0.001$) and erythrocyte MDA levels in moderate and severe activity groups of RA were significantly higher ($p<0.001$). Whereas plasma SOD levels showed no significant change ($p=0.241$), erythrocyte SOD levels were significantly increased in RA patients ($p<0.001$). Although increase in erythrocyte SOD activity in mild RA was not significant, it was significant in moderate and severe RA ($p=0.002$ and $p<0.001$, respectively).

Conclusion: These results suggest that increased plasma and erythrocyte MDA levels in RA patients, especially in those with severe activity, indicate an increased oxidative stress due to inflammation. Nonetheless, an increase particularly in erythrocyte SOD activity in moderate and severe RA patients suggests that the cellular antioxidant system might counterbalance the oxidant status in RA patients.

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Özet

Amaç: Son yıllarda romatoid artrit (RA) gibi inflamatuvar artritlerin patogenezinde reaktif oksijen metabolitlerinin rolüne ilgi oldukça artmıştır. Bizim bu çalışmamızdaki amaçlarımız RA hastalarında plazma ve özellikle eritrositlerdeki oksidan ve antioksidan statüyü araştırmak ve bu statünün hastalık aktivite indeksi (DAS) 28'e göre ılımlı, orta ve şiddetli olarak ayrılan hastalık aktivitesi ile ilişkisini belirlemektir.

Yöntem ve Gereçler: Elli RA hastası ve 26 kontrolden alınan açlık kan örneklerinde plazmada ve eritrosit içinde malondialdehit (MDA), ksantin oksidaz (XO), ve superoksit dismutaz (SOD) değerlerine bakıldı. Hastalık aktivitesinin değerlendirilmesinde ise Hastalık Aktivite Skoru (DAS)-28 kullanıldı.

Bulgular: Romatoid artrit hastalarında MDA ve XO'nun plazma seviyeleri ile MDA ve SOD'nin eritrosit seviyeleri kontrol grubundan anlamlı derecede fazla idi. Her ne kadar MDA'nın plazma seviyeleri ılımlı ve orta, eritrosit seviyeleri ise ılımlı aktivite grubunda kontrollerden farklı değilse de ($p>0.05$) plazma seviyeleri şiddetli ($p=0.001$), eritrosit seviyeleri hem orta ($p<0.001$) hem de şiddetli RA hastalarında ($p<0.001$) anlamlı olarak yüksekti. RA hastalarında plazma SOD seviyeleri anlamlı yükseklik göstermese de ($p=0.241$) eritrosit SOD seviyelerinde anlamlı bir artış vardı ($p<0,001$). Eritrosit SOD aktivitesindeki bu artış ılımlı RA'da anlamlı olmasa da orta ve şiddetli RA'da oldukça anlamlıydı (sırasıyla $p=0.002$ and $p<0.001$).

Sonuç: Çalışmamızda bulduğumuz sonuçlar özellikle şiddetli hastalık aktivitesi bulunan RA hastalarında artan plazma ve eritrosit MDA seviyelerinin inflamasyona bağlı olarak oksidatif stresi artırdığını göstermektedir. Bununla birlikte özellikle orta ve şiddetli aktivitesi olan RA hastalarındaki eritrosit SOD aktivitesindeki artış ile bu strese en azından hücresel seviyede karşı koymaya çalışıldığını öne sürebiliriz.

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Anahtar sözcükler: Antioksidanlar, hastalık aktivitesi, romatoid artrit

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Introduction

Rheumatoid arthritis (RA) is a chronic, progressive autoimmune disorder of unknown etiology with symmetric erosive synovitis and sometimes with multisystem involvement (1). It is characterized by a chronic hypertrophic synovitis leading to destruction of connective tissue and functional damage of cartilage and bone structures (2). Long-term outcome of this disease is characterized by significant morbidity, loss of functional capacity and increased mortality (3).

The causes of RA have not been completely elucidated. Oxidative stress can play an important role in the pathogenesis of RA. Acute and chronic oxidant stress to the vascular endothelium is a serious causative factor of vascular endothelial dysfunction and plays an important role in the pathophysiology of some diseases, including diabetes (4), panic disorder (5) and inflammatory bowel diseases (6). In recent years, increasing attention has been given to the role of reactive oxygen metabolites in the pathogenesis of inflammatory disease such as RA. Increased activity of free radicals, the unstable molecules associated with cell damage, is theorized to underlie the mucosal injury commonly seen in the various inflammatory diseases (7).

It has been suggested by several researchers that enzymatic and/or non-enzymatic antioxidant systems are impaired in RA, and that RA patients are thus exposed to oxidant stress (8, 9). Different activities of oxidant and antioxidant enzymes, namely xanthine oxidase (XO) and superoxide dismutase (SOD), have been reported (10, 11). It has also been supposed that RA patients are more prone to lipid peroxidation because of the reduced antioxidant defense system (12).

In this study, plasma and erythrocyte levels of antioxidant and oxygen-derived free radical status of patients with RA were investigated and compared with that of age- and sex-matched healthy controls. Additionally, relationships between these parameters and disease activity indices were assessed.

Materials and Methods

Patients and Disease Activity Assessment

Fasting blood samples were obtained from 50 RA patients and 26 healthy subjects. This study was approved by the Local Ethical Committee, and all the subjects gave their informed consent prior to their inclusion in the study. All patients were diagnosed as having RA according to the American Rheumatism Association criteria of 1987 (13). As the aim of our study was not to determine the specific effects of various treatments or dosage regimens, we only investigated the relationship of disease activity with plasma and erythrocyte oxidant/antioxidant levels. Neither control subjects nor RA patients had clinical or laboratory evidence of any disease such as diabetes mellitus or thyroid disease or any infectious disease that might have affected the parameters to be measured, and none of the subjects was a smoker or taking alcohol or an antioxidant agent (e.g. vitamins E, C). All patients were using disease modifying anti-rheumatic drugs (24 methotrexate (MTX), 17 MTX and sulfasalazine combination, 5 MTX and chloroquine combination, and 4 leflunomide). Fourteen patients who were using only MTX were taking low doses (<10 mg/day) of steroid. None of the patients was using biological agents. The demographic parameters of RA patients and controls are given in Table 1.

To evaluate the activity of RA, Disease Activity Score (DAS)28 was used. DAS28 was calculated using the following formula (14): DAS28 =0.56 x √ (tender 28)+0.28 x √ (swollen 28)+0.70xLn (ESR) + 0.014 x GH (ESR: erythrocyte sedimentation rate, mm/h), GH: Global Health measured on a Visual Analogue Scale; the number of swollen joints and tender joints were assessed using 28-joint counts) (15). A DAS28 value below 3.2, a value between 3.2 and 5.1, and a value above 5.1 indicated mild, moderate, and severe disease activity, respectively (14).

Biochemical Analysis

Sample collection and preparation

Blood samples obtained after overnight fasting were collected from the antecubital vein in polystyrene tubes containing potassium EDTA. The blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to remove plasma and then the buffy coat on the erythrocyte sediment was separated carefully. Erythrocyte sediment was washed four times with 10-fold isotonic NaCl solution to remove plasma remnant. After each procedure, erythrocyte-saline mixture was centrifuged at 3000 rpm for 10 min at 4°C. Aliquots of the samples were transferred into polyethylene tubes to be used in the assay of MDA, SOD and XO levels. ESR and serum C-reactive protein (CRP) levels were estimated within two hours. Plasma and erythrocyte sediment samples were stored for 30 days at –80 °C until malondialdehyde (MDA), SOD and XO analyses were performed. After they were thawed, erythrocyte sediments were treated with four-fold ice-cold deionized water to obtain hemolysate.

Enzymes, chemicals and instruments

Xanthine oxidase, xanthine, nitroblue tetrazolium (NBT), thiobarbituric acid, 1.1, 3.3-tetramethoxy propane, adenosine, phenol, Na nitroprusside, uric acid, CuCl2, bovine serum albumin, H_2O_2, EDTA, Na_2CO_3, $(NH_4)_2SO_4$, chloroform, ethanol, NaCl, KH_2PO_4, and $Na_2HPO_4{\cdot}2H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO) and Merck (Germany). Shimadzu UV-1601 UV/VIS spectrophotometer was used to measure all the parameters studied.

CRP and ESR determination

Erythrocyte sedimentation rate and CRP were determined in whole blood and serum aliquots,

respectively. ESR was determined according to the Westergren method and CRP by a nephelometric method (Dade Behring BN II, USA).

MDA determination

Malondialdehyde levels in plasma were measured with the thiobarbituric acid reaction by the method of Placer et al. (16) as described in previous studies (17). The values of MDA were expressed as nmol/ml^{-1} for plasma and nmol/g Hb for erythrocyte.

XO activity determination

Xanthine oxidase (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm (18). A calibration curve was constructed by using 10-50 milliunits/mL concentrations of standard XO solutions (Sigma X-1875). One unit of activity was defined as 1 µmol uric acid formed per minute at 37°C, pH 7.5. Results were expressed as U/ml for plasma in 293 nm wavelength.

SOD activity determination

Total SOD activity (Cu/Zn and Mn) was determined according to the method of Sun et al. (19) and a slightly modified method by Durak et al. (20). The principle of the method is based on the inhibition of NBT reduction by the xanthine/XO system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 mL ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged at 4000 *g* for 30 min at 4°C. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the rate of NBT reduction. Activity was expressed as U/ml for plasma and U/g Hb for erythrocyte in 560 nm wavelength.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (Versions 6.0 and 10.0, SPSS Inc., Chicago, IL, USA). Distribution of the groups was analyzed with one sample Kolmogorov-Smirnov test. Neither group showed normal distribution, so the nonparametric Bonferroni-adjusted Mann-Whitney U test was performed on the biochemical variables data to examine differences among the groups. Bivariate comparisons were examined using Spearman's ρ correlation test. A $p \leq 0.05$ was accepted as statistically significant.

Results

The clinical features and laboratory findings of patients and control subjects are shown in Table 1. There were no significant differences according to age or sex between the patients and controls (p>0.05).

The mean levels of ESR and CRP were significantly higher in RA patients compared to controls (30.78±17.99 mm/hr vs. 14.38±5.30 mm/hr, p<0.001; 15.12±14.98 mg/dl vs. 3.15±1.40 mg/dl, p<0.001, respectively).

The plasma levels of MDA (pMDA) and XO (pXO) and erythrocyte levels of MDA (eMDA) and activity of SOD (eSOD) were significantly higher in RA patients than in control subjects (p=0.009, p=0.001, p<0.001, p<0.001, respectively). While eSOD activity was significantly higher in RA patients, pSOD activity showed no significant change between the groups (p=0.241).

When we compared the groups, while plasma levels of MDA and XO and erythrocyte levels of MDA and activity of SOD were significantly different in RA groups, there was no significant difference in the plasma activity of SOD (p values 0.018, 0.003, <0.001, <0.001, 0.406, respectively).

Although increases in pMDA levels in mild and moderate and eMDA levels in mild activity groups of RA were not significant (p>0.05), pMDA levels in severe (p=0.001) and eMDA levels in moderate and severe activity groups of RA were significantly higher (p<0.001). Whereas pSOD activity showed no significant change (p=0.241), eSOD levels significantly increased in RA patients (p<0.001). Although increase in eSOD activity in mild RA was not significant, it was significant in moderate and severe RA (p=0.002 and p<0.001, respectively) (Table 2).

Discussion

Rheumatoid arthritis is a chronic relapsing immuno-inflammatory multisystem disease with predominant synovial proliferation and destruction of the articular cartilage and bone (28). The etiopathogenesis of RA remains

Table 1. Plasma and erythrocyte oxidant and antioxidant status in rheumatoid arthritis and control groups

	Control (n=26)	Patient (n=50)	p
Age (years)	56 (35-68)	53 (20-74)	>0.05
N (F/M)	20/6	41/9	>0.05
Duration of disease (years)		8 (1-45)	
pMDA (nmol/ml)	0.87 (0.60-1.15)	0.98 (0.60-1.47)	0.009 *
pXO (U/ml)	1.04 (0.71-2.28)	1.61 (0.84-3.27)	0.001 *
pSOD (U/ml)	4.70 (0.49-10.16)	4.52 (0.45-8.77)	0.241
eMDA (nmol/g Hb)	17.25 (4.81-39.02)	28.40 11.62-56.89	<0.001 *
eSOD (U/g Hb)	1610.84 758.02-4929.46	3087.89 689.57-5166.64	<0.001 *

Values are given as the median (minimum-maximum) (Mann-Whitney U test)
p: Plasma, e: Erythrocyte, MDA: Malondialdehyde, XO: Xanthine oxidase, SOD: Superoxide dismutase, F/M: Female/Male

Table 2. B coefficients and odds ratios of variables in the final regression model

	Control (n=26)	Mild RA (n=14)	Moderate RA (n=15)	P[a]	Severe RA (n=21)	p[b]
Age (years)	56	48	51	0.327	57	0.506
	(35-68)	(35-74)	(31-68)		(20-70)	
DAS28		2.62	4.52		6.07	
		(1.80-3.19)	(3.84-5.02)		(5.15-7.16)	
ESR (mm/hr)	15	21	25	0.001*	35	<0.001*
	(10-35)	(10-51)	(8-75)		(13-86)	
CRP (mg/dl)	3.2	7.05	11	<0.001*	16	<0.001*
	(0.5-5.15)	(2.1-32)	(3.1-34)		(3.1-81)	
pMDA (nmol/ml)	0.87	0.95	0.91	0.221	1.04	0.001*
	(0.60-1.15)	(0.60-1.33)	(0.60-1.34)		(0.75-1.47)	
pXO (U/ml)	1.04	1.44	1.67	0.007*	1.84	0.002*
	(0.71-2.28)	(0.96-3.22)	(0.97-2.23)		(0.84-3.27)	
pSOD (U/ml)	4.70	4.71	4.58	0.478	4.34	0.149
	(0.49-10.16)	(0.31-6.61)	(0.96-6.40)		(0.45-8.77)	
eMDA (nmol/g Hb)	17.25	21.14	33.45	<0.001*	34.37	<0.001*
	(4.81-39.02)	(11.80-45.51)	(11.62-41.25)		(14.55-56.89)	
eSOD (U/g Hb)	1610.84	2746.16	2968.73	0.002*	3304.14	<0.001*
	(758.02-4929.37)	(689.57-4955.60)	(1362.22-5061.66)		(1549.08-5166.64)	

p[a] statistical significance between moderate and control groups p<0.008

p[b] statistical significance between severe and control groups p<0.008

(Bonferroni-adjusted Mann-Whitney U test)

Values are given as the median (minimum-maximum)

DAS: Disease Activity Score, ESR: Erythrocyte sedimentation rate, CRP: C-reactive protein, MDA: Malondialdehyde, XO: Xanthine oxidase, SOD: Superoxide dismutase, p: Plasma, e: Erythrocyte

obscure despite extensive research. The pathogenesis of RA is multifactorial and recent research has implicated oxygen free radicals as a mediator of tissue damage (21). It has been shown that, especially in RA, monocytes produce 2.7 times more oxygen radicals than controls (22). Over-production of reactive oxygen species leads to lipid peroxidation and destroys the antioxidant defense systems (12). Based on this hypothesis, several studies have been focused on oxidative stress in RA (8, 9, 12).

Lipid peroxidation can start by the attacks of free radicals on lipids. After a series of propagation reactions and hydroperoxide formation, these active substances decompose various end products, like MDA. It has been supposed that blood samples from patients with RA are more prone to lipid peroxidation owing to an impaired antioxidant defense system (12). In our study, pMDA and eMDA levels were found to be significantly elevated in the patients with RA compared to controls (Table 1). This is in agreement with other studies in which higher MDA levels have been reported in patients with RA (9, 23, 24). When we look at this finding with respect to disease activity levels, a significant increase was found in plasma levels of severe RA and erythrocyte levels of moderate and severe RA. We can suggest that oxidant injury is related to disease activity.

It is possible that disordered mitochondrial oxidative phosphorylation (25) leads to diminished production of toxic free radicals through the xanthine/XO system. These changes lead to accumulation of adenosine and of its breakdown products, xanthine and hypoxanthine, which can act as substrates for XO (26). Oxygen radical production in this setting is thus thought to be in part the result of the action of XO (25). In our study, pXO levels were high in RA patients; this increase was observed particularly in moderate and severe groups.

Mammalian cells have developed antioxidant defense systems to prevent oxidative damage and to allow survival in an aerobic environment (27). These systems consist of nonenzymatic antioxidants-including nonprotein antioxidants with low molecular weights (vitamins A and E, beta-carotene, uric acid) and of enzymes such as SOD, catalase, glutathione peroxidase, and glutathione reductase. The activity of SOD, a catalyst for dismutation of superoxide radicals into H_2O_2 and into molecular oxygen, protects cell and tissues from superoxide radicals and other peroxides (28). In several studies, lower activity of SOD (29) and no change in SOD (30) were shown. In our study, we found a significant increase in the activity of eSOD in severely active RA patients compared to RA patients with mild or moderate

disease activity; however, we found no significant change in plasma SOD (pSOD) activity levels. With regard to these findings, it can be suggested that the antioxidant defense system protects tissue from oxidant injury in moderate and severe patients but at the cellular rather than plasma level.

Erythrocytes may be important in regulating oxidant reactions in the surrounding medium, thereby preventing free-radical-mediated cytotoxicity (31). However, the relationship of disease activity of RA with erythrocyte activity of SOD and levels of MDA and XO is not clear. Our findings suggested that the levels of eMDA and the activity of eSOD were increased in RA patients, consistent with most of the previous studies (23, 32). On the other hand, Banford et al. (33) reported a significant decrease in eSOD activity in RA patients. In our study, the levels of eMDA and eSOD were significantly higher in the patients with moderate or severe activity. These findings suggest that cellular enzymatic antioxidant defense system precedes the equilibrium of oxidant and antioxidant status in RA patients.

The limitation of this study is, when we separated patients into groups according to disease activity, the groups were small. Therefore, studies with greater numbers of patients should be done in the future.

Conclusion

In conclusion, increased oxidative stress in patients with RA probably depends on their inflammatory response. Our results indicated that oxidative stress and lipid peroxidation were accelerated in patients with RA. Whereas this acceleration was highest in severe disease, the antioxidant system in erythrocytes is also significantly high. This increase suggests that cellular enzymatic antioxidant defense system precedes the equilibrium of oxidant and antioxidant status in RA patients.

In this regard, we highlight some therapeutic aspects, such as using high potential antioxidants that potentiate antioxidant defense mechanisms and reduce peroxidation, which may be beneficial in the management of RA.

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Conflict of Interest

None declared.

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