

Original research-Orijinal araştırma

In vivo effect of colchicine, colchicine and anti-aggregating agent on protein oxidation in Behcet's Disease

Behçet hastalığında kolşisin, kolşisin ve antiagregan ajanın protein oksidasyonu üzerine in vivo etkileri

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Abstract

Aim. Behcet's Disease is a chronic, systemic inflammatory disease. Oxidative stress related to plasma protein modifications may have an important role in the progression of Behcet's Disease. In this study the effect of oxidative damage during the course of Behcet's disease on plasma proteins and the effect of colchicine and anti-aggregating agents on prevention or depression of oxidative damage was evaluated by plasma protein characterization and quantitation. **Methods.** 45 Behcet's Disease and 40 control subjects were included in this study. Study groups are determined as Group I: in inactive stage receiving no drugs, Group II: active stage receiving only colchicine (1.0-2.0 mg/day), and Group III: in active stage receiving colchicine and antiaggregating agent (acetyl salicylic acid 100 mg/day). Plasma total protein, plasma carbonyl content, plasma total thiol levels were determined spectrophotometrically. Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were evaluated by conventional methods. The protein peak areas were analyzed through high pressure liquid chromatography (HPLC). **Results.** Plasma protein levels were significantly higher in Group I and Group II compared to controls. Plasma carbonyl levels were higher in Group I, II and III than the control groups. After colchicine ve antiaggregating agent treatment in patient groups plasma carbonyl and were found significantly lower whereas thiol levels were found significantly higher in Group III than the other patient groups. Respons of plasma CRP and ESR levels to treatment in Group III were significantly positive compared to the other groups. Plasma protein fragmentation was not dedected in Behcet's diseases. **Conclusion.** The plasma protein data which developed upon oxidative stress is very valuable in physiopathology of Behcet's Disease. When the relation between severity of the disease and the extent of oxidative stress is considered, multicentral and longterm clinical follow ups could be proposed.

Keywords: Behçet's disease, colchicine, antiaggregating agent

Özet

Amaç. Behçet hastalığı (BH), kronik, sistemik bir enflamatuvar hastalıktır. Oksidatif stres plazma protein modifikasyonlarıyla ilişkilidir ve Behçet hastalığının gelişiminde önemli rol oynayabilir. Bu çalışmada, Behçet hastalığında geliştiği düşünülen oksidatif hasarın plazma proteinlerine etkisinin belirlenmesi ve tedavide kullanılan kolşisin ve antiagregan ilaçların oksidatif hasara/cevaba etkisinin protein kantitasyonu yapılarak değerlendirilmesi amaçlanmıştır. **Yöntem.** Çalışmaya 45 Behçet hastası ve 40 kontrol grubu dahil edildi. Çalışma grupları, Grup I: hiç ilaç kullanmayan inaktif durumdaki hastalar; Grup II, yalnız kolşisin kullanan (1,0-2,0 mg/gün) ve Grup III, aktif durumdaki kolşisin ve antiagregan (asetil salisilik asid 100 mg/gün) kullanan hastalar. Plazma total protein, plazma karbonil, total tiyol seviyeleri, ESR ve CRP seviyeleri belirlendi. Protein pik alanları yüksek basınçlı sıvı kromatografisi ile analiz edildi. **Bulgular.** Plazma protein düzeyleri kontrollarla karşılaştırıldığında Grup I ve II'de anlamlı derecede yüksekti. Plazma karbonil düzeyleri kontrollere göre Grup I, II ve III'de anlamlı derecede

yüksekti. Kolşisin ve antiagregan tedavisi sonrası Grup III'de karbonil düzeyleri anlamlı derecede düşerken plazma tiyol düzeyleri anlamlı derecede yüksek bulundu. Plazma CRP ve ESR düzeylerinin tedaviye cevapları Grup III'de diğer gruplarla karşılaştırıldığında anlamlı derecede pozitif bulundu. Plazma protein fragmentasyonu Behçet Hastalarında tespit edilmedi. **Sonuç.** Oksidatif strese bağlı olarak gelişen plazma protein modifikasyon verileri Behçet hastalığının patofizyolojisinde çok değerli olabilir. Hastalığın şiddeti ve oksidatif stresin yaygınlığı arasındaki ilişki de gözönüne alındığında çok merkezli ve uzun dönem klinik takiplerin olduğu çalışmaların yapılması gerekmektedir.

Anahtar sözcükler: Behçet hastalığı, kolşisin, anti-agregan ilaç

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Introduction

Behcet's Disease was first described by Dr. Hulusi Behçet, Turkish dermatologist. It is a multisystemic disease with oral and genital aphthous ulcers, arthritis, skin and eye lesions where gastrointestinal system and central nervous system are involved [1]. Although the etiopathology of this system is not clearly described, it is proposed that bacterial and viral infections [2, 3], immunological defects [4] and genetic factors [5] play roles but there is no specific treatment protocol since the treatment may vary with the severity of the affected organs and also there is lack of multicenter, multidisciplinary, long term and controlled works. Colchicine is one of the drugs used most frequently in Behcet's disease treatment. Corticosteroids, non steroidal, anti-inflammatory, immunosuppressive and fibrinolytic agents are in the treatment alternatives. In recent years, the role of free radicals in the etiopathogenesis of a variety of diseases and in tissue damage has caused great interest in medical field. The oxidative stress is described as increase in exposure to increased oxidants and/or decrease in antioxidant capacities. In normal conditions there is a balance between antioxidation defense system and oxidative system. As a result of the excess production of free oxygen radicals, the defense system is impaired and oxidative damage is produced [6, 7]. Behcet's disease shows attacks to many organs due to its multisystemic character. There are various studies reporting the effect of oxidative stress which results due to increase in free radical production in this disease [8-10]. But, like in many diseases, it is not well described yet whether the oxidative stress is the cause of the disease, or whether it is the result of the disease. Although research on the oxidative stress in various diseases concentrates on the identification of specifically oxidized proteins, in Behcet's disease there are limited clinical studies which reflect the quantitative protein inactivation and treatment strategies in patient population [11].

In this work the effect of the oxidative damage which is thought to be produced in Behcet's disease, on plasma proteins and during the course of the disease, treatment with colchicine and anti-aggregating drugs was investigated by protein quantitation and characterization.

Material and methods

Study subjects

This study is performed together with by Cyprus University, Faculty of Pharmacy, Department of Biochemistry; Marmara University, Faculty of Pharmacy, Department of Biochemistry and Marmara University, Medical School, Department of Rheumatology. Written informed consents, as approved by the Ethics Committee of Marmara University

Medical Faculty, Istanbul, Turkey were obtained for individual cases. Patients with Behcet's Disease (female: 24, male: 21) and normal healthy volunteers (female: 20, male: 20) were included in this study. The eligible patients were of 35 ± 17 years of age for males, and it was 36 ± 16 for females. The average age of the control group was 32 ± 13 years. Behcet's Disease patients who had active disease were selected according to the diagnostic criteria for clinical remission of the International Study Group (ISG) [12]. Working groups are divided as Group I: in inactive stage receiving no drugs, n: 8; Group II: in active stage receiving only colchicine (1.0-2.0 mg/day), n: 15; and Group III: in active stage receiving colchicine and antiagregating agent (acetyl salicylic acid 100 mg/day), n: 22. All groups were followed up for three months. Behcet's Disease patients in active stage were selected from patients who had systemic involvement. On the other hand, the patients in inactive stage had only folliculitis and oral aphthous lesions. Uncontrolled medical conditions such as diabetes, cancer, and patients using antioxidants or who smoke were excluded.

Assay procedures

Blood was obtained by venipuncture. Control plasma was prepared from participants who reported to have no history of Behcet's Disease. To obtain plasma, blood was obtained in 0.12 M trisodium citrate (9:1 ratio, v/v). Citrated blood samples were centrifuged at $1,500 \times g$ for 10 min at 4°C and supernatants were divided into aliquots for storage at -80°C until further use. Erythrocyte sedimentation rate (ESR) was determined by the method of Gambino et al. [14].

Protein and carbonyl levels

Total protein content in plasma was measured by Folin-phenol reagent as described by Lowry et al. [14] while using BSA as the standard. Plasma C-reactive protein (CRP) levels in each specimen (in mg/L) were quantified by a commercially available assay as described [15]. Protein carbonyls were estimated using the method of Levine et al. [16] with slight modifications. Briefly, 0.5 mL plasma (1 mg/mL) were treated with 0.5 mL trichloro acetic acid (TCA, 20 % v/v) at room temperature for 10 min, and centrifuged at $4,000 \times g$. The pellet was treated with 0.5 mL of 10 mM DNPH in 2 M HCl or with 0.5 ml of 2 M HCl alone for the blank. Samples were incubated for 30 min at room temperature in the dark, and then treated with 20% TCA, and centrifuged at $4,000 \times g$. The pellet was washed three times in ethanol/ethyl acetate (v/v); and 1.5 mL of 1 M NaOH was added to pellet followed by incubation at 37°C for 15 min. Carbonyl concentrations were determined utilizing molar absorption coefficient of $\epsilon_{370} = 22,000 \text{ M}^{-1}\text{cm}^{-1}$ using a Shimadzu UV-spectrophotometer and expressed as nanomoles of carbonyls per milligram protein.

Plasma thiol levels

Plasma thiol levels were measured by Ellman reagent (5, 5'-ditiobis 2-nitro-benzoic acid-DNTB) as described [17]. Samples were centrifuged at $3,000 \times g$ for 5 min at room temperature. Top phases were decanted and thiol level of all samples were determined by utilizing molar absorption coefficient ($14,100 \text{ M}^{-1}\text{cm}^{-1}$) using a Shimadzu UV-spectrophotometer and expressed as micromole.

HPLC analysis

Plasma low molecular weight (MW) protein fractions were detected on an Agilent 1,100 Series HPLC system equipped with G1315A model of diod detector after 50-fold dilution of specimens. Protein-Pack-125 column was equilibrated with 0.1 M phosphate buffer ($\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$), pH 7.4. Samples were eluted at the flow rate of 1 mL/min and detected by their absorbance at 280 nm, and the peak areas were recorded. MW of protein standards were; myosin:205 kD; β -galactosidase:116 kD; phosphorylase b:97 kD; bovine albumine:66 kD; egg albumin :45 kD; and carbonic anhydrase:29 kDs. All samples were assayed at least in duplicates [18].

Statistical analyses

The continuous variables were expressed as mean \pm standard deviation (SD). Comparisons between the controls vs. drug and nondrug receiving patient groups were analyzed by ANOVA (post-hoc Tukey's test). Statistical significance was expressed when the achieved p values were <0.05 .

Results

In this work, 45 Behcet's Disease patients, 8 in inactive stage and 37 in active stage, and 40 control subjects were examined with respect to plasma protein oxidation and inflammation. The patient groups were divided as patients receiving no drug (Group I), receiving colchicine (1.0-2.0 mg/day, Group II) and receiving colchicine together with anti-aggregating drug (acetyl salicylic acid, 100 mg/day) and healthy controls. Plasma protein levels, plasma carbonyl concentrations, thiol levels were evaluated spectrophotometrically, CRP and ESR values were determined by conventional methods and the protein peak area changes in plasma proteins were determined by HPLC. Plasma protein levels were significantly higher in Group I and in Group II compared to controls ($p<0.001$) whereas Group III values were not significantly different from the controls. When the patient groups were compared, Group II had protein levels higher than Group I and Group III, but Group III was significantly lower than both Group I and Group II ($p<0.001$). This showed that colchicine treatment caused the highest effect on plasma protein concentration (Group III) than Group II and the controls. When the plasma carbonyl levels were considered Group I, II and III had significantly higher levels than the controls. When the comparison was made between the patient groups, carbonyl content in Group III was significantly lower than Group I and Group II ($p<0.01$). Plasma thiol levels in 3 Groups were significantly lower than the controls ($p<0.001$). In between the patient groups, Group III thiol levels were higher than Group I and II ($p<0.01$). CRP and ESR levels in all of the groups were significantly higher than the controls ($p<0.001$). But CRP and ESR levels were significantly lower in Group III than Group I and Group II ($p<0.01$) (Table 1).

Table1. Oxidative stress and inflammation markers in control and Behcet's Disease patients.

Parameters	Control	Group I	Group II	Group III
Plasma protein (mg/ml)	6.8 \pm 0.3	7.75 \pm 1.36 ^b	8.82 \pm 2.15 ^{b#}	7.05 \pm 1.31 ^{a#}
Plasma carbonyl levels (nmol/mg)	0.40 \pm 0.05	0.74 \pm 0.34 ^b	0.69 \pm 0.21 ^b	0.62 \pm 0.1 ^{a#}
Thiol levels (μ mol/L)	526 \pm 24	262 \pm 13 ^c	273 \pm 14 ^c	298 \pm 16 ^{c#}
CRP (mg/L)	1.96 \pm 0.52	36.70 \pm 7.43 ^c	26.28 \pm 3.24 ^c	19.16 \pm 3.89 ^{c#}
ESR (mm/saat)	8.54 \pm 2.43	21.85 \pm 9.61 ^c	19.24 \pm 11.48 ^c	14.21 \pm 17.28 ^{c#}

CRP: C-reactif protein; **ESR:** Erythrocyte sedimentation rate

^a $p<0.05$ significance to the control

^b $p<0.01$ significance to the control

^c $p<0.001$ significance to the control

[#]significance in between groups

In plasma HPLC analysis the protein peak areas with approximately 7.40 min Rt (retention time) corresponding to 94 kDa molecular weight protein were significantly high in all patients groups compared to the controls. In comparison between the groups, Group I and III peak areas were found to have the lowest values than Group II. On the other hand in Group II and III, significant increases in the protein areas with the retention time 11. min were detected and evaluation between groups this protein peak had the lowest area in Group I. The plasma protein with Rt: 12 min had significantly low values of peak areas in Group I, II III compared to the control. In the evaluation between the patient groups, Group II had lower peak areas than Group I and III (Table 2).

Table 2. Retention time (Rt: min), peak areas (%), molecular weight (MW, kD) of the plasma proteins of the control and Behcet's Disease patients Groups (Group I, II, III) by HPLC analysis.

Control			Grup I			Gorup II			Group III		
Rt (min)	Area (%)	MW (kD)	Rt (min)	Area (%)	MW (kD)	Rt (min)	Area (%)	MW (kD)	Rt (min)	Area (%)	MW (kD)
4.88±0,00	19.07	>250	4.89±0.02	15.84	>250	4.88±0.01	18.08	>250	4.87±0.00	18.07	>250
7.38±0,00	24.27	94	7.37±0.06	30.45 ^a	94	7.43±0.06	31.57 ^a	94	7.38±0.05	28.78 ^{a#}	94
8.27±0,00	49.59	60	8.13±0.01	44.50	63	8.16±0.07	43.50	64	8.18±0.08	44.38	63
11.11±0,00	1.19	14	11.06±0.13	1.09 [#]	15	11.01±0.06	1.38 ^a	15	11.02±0.07	1.50 ^b	15
12.06±0,00	4.36	9	12.04±0.01	3.41 ^{a#}	9	11.94±0.04	2.04 ^c	9	12±0.04	2.28 ^c	9

^ap<0.05 significance to the control
^bp<0.01 significance to the control
^cp<0.001 significance to the control
[#]significance in between groups

Discussion

It was proposed that in Behcet's Disease oxidant-antioxidant balance was disrupted and increased production of free radicals was proposed to play a role in pathogenesis of the disease [8-10]. Free radicals affect cells' important components, such as lipids, proteins, DNA, carbohydrates and enzymes and cause functional changes. It is well known that proteins are more sensitive to free radicals and therefore, this oxidative protein damage is more lasting and cytotoxic [19]. Colchicine, is a natural toxic product which is used as a prophylactic agent and it is a most commonly prescribed drug for Behcet's Disease treatment. Colchicine is a corticosteroid and it prevents disease to be more severe by inhibiting neutrophil chemotaxis and reducing number of cells that are collected at inflammatory area [20]. In addition, it was reported that colchicine increase cleaning activity of free radicals at polymorphonuclear cells. In other words, it becomes protective by blocking free radical production because of phagocytosis. Therefore, cells's free radical production is reduced even though there is no direct increase in superoxide dismutase. It was documented that tissue damage at Behcet's Disease patients was caused by increased oxygen concentration that was produced by polymorph nuclear neutrophils [21]. In our study, we showed that colchicine alone is not enough to control inflammation markers CRP and ESR levels. However, when data from colchicine treated patient group was compared with other two groups there was relative reduction in inflammation markers. In addition, the anti-aggregating agent, aspirin, inhibits cyclooxygenase pathway when used as a co-treatment. Inflammation and neutrophil chemotaxis reduced by controlling cyclooxygenase pathway [21]. On the other hand, increase in neutrophil chemotaxis was reported in Behcet's Disease. Parallel to increase in neutrophils, significant amount of superoxide production by neutrophils was observed in active Behcet's Disease patients [22]. Protein carbonyl levels, highly stable and created as a result of metal catalyzed modification of proteins, are used extensively for clinical diagnosis of protein oxidation. Because of chemical resistance of protein carbonyl levels compared to lipid peroxidation products, it is more appropriate parameter for laboratory diagnostics. Our data shows that, protein carbonyl levels were significantly increased in untreated group compared to control group. On the other hand, colchicine plus anti-aggregant treatment group (Group III) had significant decrease in protein carbonyl levels compared to other groups. Similarly, Kaya and colleagues proposed that high levels of superoxide production resulted in protein oxidation. In addition, they proposed that pyrolyzed proteins, produced as a result of reaction between amino groups of proteins and lipid peroxidation products, can be used as a marker for oxidative stress in this disease [11]. In another study, it was shown that increased levels of MDA and protein carbonyl

levels were reduced by colchicine treatment [23, 24]. Since reduction in plasma protein carbonyl levels were observed only in colchicine and anti-aggregant treatment groups. Further studies on drug interactions and changes in protein levels via oxidation power of the disease are necessary. While total plasma protein concentration of colchicine and anti-aggregating treatment group (Group III) was similar to control group, protein carbonyl levels were at lowest level. These results showed similarity to our previous findings on primary osteoarthritis patients where it was reported that when total protein concentration was at lowest level, protein carbonyl levels concentration was at its highest level [25]. Antioxidant immune defense system of Behcet's Disease patients was disrupted [26]. In our study we showed: (i) the link between reduction in antioxidant immune defense system effectiveness and oxidant stress level; (ii) that colchicine relatively supports immune defense system; and (iii) agents co-treated with colchicine have positive effect on immune defense system; (iv) on the other hand, reduction in immunoglobulin synthesis ratio was documented in plasma of the same groups. Lowest thiol concentration in Group I was found to be parallel to high carbonyl levels in the same group. Although there are limited number of reported studies in the literature showing low thiol levels observed in Behcet's Disease patients, the effect of Behcet's Disease treatment on thiol levels have not been studied previously. However, decrease in the level of glutathione (GSH), the most thiol group containing antioxidant enzymes, in plasma and serum of Behcet's Disease patients was demonstrated [27]. In addition, increase in total oxidant intensity and decrease in total anti-oxidant capacity was reported in these patients [28, 29]. In Behcet's Disease patients reduction in Vitamins E, C, B1, B2 and flavin mononucleotide levels was proposed [30]. Evereklioglu and colleagues reported increase in neutrophil, acute phase marker numbers and erythrocyte sedimentation speed in both active and inactive periods of the disease in addition to increase in IL-6, IL-8 and TNF- α levels [31]. Similarly, Levamisole and colchicine treatment was shown to reduce levels of these cytokines [23]. Free radicals, produced during oxidative stress, oxidize thiol groups of proteins. In order to reduce and use these thiol groups against oxidative damage GSH is required. However, decrease in GSH during oxidative stress results in decrease in reduction [329]. In conjunction with treatment, use of antioxidants, such as Vitamin E, was shown to increase antioxidant capacity in Behcet's Disease patients [11, 27]. Fragmentation of plasma proteins or change in plasma protein synthesis speed, as a result of intensity of oxidative strength, can be quantitatively measured by HPLC [18, 25, 33]. In other word, fragmentation of proteins enhances in pathological conditions where oxidative strength reduces. However, oxidative stress dependent fragmentation of plasma proteins was not observed in our study on Behcet's Disease patients. On the other hand, reduction in immunoglobulin synthesis ratio was documented in plasma of the same patient group. As a result of plasma protein analysis we found significant increase in area of α -1 antitrypsin in all three patient groups compared to controls. Previous findings showing increase in acute phase reactants α -1 antitrypsin and alfa-2-microglobulin at Behcet's Disease further supports our findings [31]. Although, Methotrexate and sulphasalazine is accepted as effective treatments by many clinicians, these observations lack control studies. It is thought that, use of corticosteroids with immunosuppressive agents and anticoagulant-anti-aggregants decrease postoperative repeats, and graft occlusions [34, 35]. Heat shock proteins (HSP) are possible antigen candidates for infection and immunity related to Behcet's Disease. Heat shock protein 70 (HSP 70), which plays an anti-inflammatory role, with respect to control group was found to be high in active patients. Inactive patients had lowest HSP activity than active patients [36]. We showed that peak area of plasma proteins that has 60 kDa molecular weight was not changed. Therefore, it is not possible to state that the treatment strategies applied to Behcet's Disease patients in our study results in reduction in these proteins. Accordingly, further studies on regulation of HSP synthesis in these patients are necessary.

In the present study, our findings are as follows: (i) In Behcet's disease oxidant-antioxidant equilibrium was disrupted; (ii) increased protein carbonyl levels as a result of

enhanced oxidative stress can be controlled by colchicine plus antiaggregating drug treatments; (iii) these drugs have effects on total thiol levels and increase the antioxidant capacity; (iv) On the other hand, reduction in immunoglobulin synthesis ratio was documented in plasma of the same groups. Since oxidative stress is correlated with severity of the disease, treatment period and drug selection can be suggested as key elements of therapies. On the other hand, in order to plan effective treatment strategies, there is a need for multicenter, multidisciplinary, high number of patients covered, controlled and long period clinical experiments.

Therefore, oxidative stress dependent plasma protein oxidation screening can be important in clinical physiopathology of Behçet's Disease. In addition, characterizing the effects of treatment agents on oxidation can be important in developing new treatment strategies.

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