Serum Oxidant/Antioxidant Status in Patients with Behçet’s Disease

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Abstract. The aims of this study were to assess whether the increased oxidative stress in affected tissues is reflected by serum lipid peroxidation and to check for alterations in serum levels of extracellular antioxidants and antioxidant enzyme activities in patients with Behçet’s disease (BD). Serum malondialdehyde (MDA) and ceruloplasmin (Cp) levels and CuZn-superoxide dismutase (CuZn-SOD) and glutathione peroxidase (GSH-Px) activities were increased, while serum transferrin (Trf) levels were diminished in patients with active ocular BD (n=19), inactive ocular BD (n=18), and nonocular BD (n=15), compared to healthy controls (n=20). Serum MDA levels in patients with active ocular BD and nonocular BD were significantly higher than in the inactive ocular BD group. Patients with active ocular BD also had significantly higher serum Cu-Zn SOD activities, compared to the inactive ocular BD. Erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) levels were higher in patients with active ocular BD, inactive ocular BD, and nonocular BD, compared to the control group. In addition, patients with active ocular BD and nonocular BD had significantly higher ESR and serum CRP levels, compared to the inactive ocular BD group. Serum albumin concentrations showed no significant differences among the BD patients and controls. The authors speculate that in BD patients, serum superoxide radicals may be dismutated to H2O2 by increased CuZn-SOD activity and the conversion of H2O2 to hydroxyl radical may be enhanced by iron, owing to diminished serum Trf; these mechanisms may contribute to the increased serum lipid peroxidation.

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Key words: Behcet’s disease, ceruloplasmin, transferrin, albumin, lipid peroxidation, free radicals

Introduction

Behçet’s disease (BD), first described by a Turkish physician, Hulusi Behçet, is a chronic, progressive disorder that affects many systems of the body, including the eye. The origin of this disease is unclear, but immunoregulatory abnormalities have been proposed as pathogenic mechanisms [1-3]. Common manifestations include oral aphthous ulcers, genital ulcers, skin lesions, and uveitis, which may cause blindness. Involvement of the G-I tract, central nervous system, and vascular system is less common but accounts for mortality associated with BD disease. Adult and pediatric cases occur [4,5].

Behçet’s disease is found worldwide, but there are marked geographical differences in disease expression. BD is more frequent in countries along the ancient Silk Route, which extends from eastern Asia to the Mediterranean basin. The incidence of BD cases per 100,000 population varies from 80-370 in Turkey to 13.5-20 in Saudi Arabia, Iran, Korea, Japan, China, and Oman [4,5].

Oxygen free radicals and lipid peroxides have been implicated in the pathogenesis of numerous diseases such as diabetes mellitus, cancer, rheumatoid arthritis, systemic lupus erythematosus, infectious diseases, atherosclerosis, and in aging [6-9]. Growing evidence indicates that oxidative stress is increased in BD, owing to overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses [10-12].

To control the flux of ROS, aerobic cells have developed their own defense system, the antioxidant
system, which includes enzymatic and non-enzymatic components [7]. The antioxidant system consists of low molecular weight antioxidant molecules such as glutathione (GSH) and various antioxidant enzymes. For instance, superoxide dismutase (SOD), the first line of defense against oxygen-derived free radicals, catalyses the dismutation of the superoxide anion (O$_{2}^{-}$) into H$_{2}$O$_{2}$. Glutathione peroxidase (GSH-Px) is a selenoprotein that reduces lipidic or nonlipidic hydroperoxides as well as H$_{2}$O$_{2}$ while oxidizing GSH. Oxidized glutathione (GSSG) is reduced back to GSH by glutathione reductase [13-16].

Albumin (Alb) contains 17 disulphide bridges and has a single remaining cysteine residue that is responsible for the capacity of albumin to neutralize peroxyl radicals. This property is important, since Alb plays a role in transporting free fatty acids in the serum [17]. A major contributor to the antioxidant defence system of human serum is ceruloplasmin (Cp), which acts as an antioxidant by several mechanisms: (a) inhibiting iron-dependent lipid peroxidation (Lp) and HO$^{\cdot}$ formation from H$_{2}$O$_{2}$ by its ferroxidase activity, (b) reacting with and scavenging H$_{2}$O$_{2}$ and superoxide anion, and (c) inhibiting copper-induced Lp by binding Cu$^{2+}$ ions [18]. Cp is a plasma glycoprotein that is synthesized primarily in the liver and secreted into the blood. Cp permits the incorporation of iron into transferrin (Tf) without the formation of toxic Fe products [19-21]. The process of lipid peroxidation involves oxidative conversion of polyunsaturated fatty acids to products such as malondialdehyde (MDA), which is usually measured as thiobarbituric acid reactive substances (TBARS), or as lipid peroxides [22].

**Patients and Methods**

A total of 52 BD patients (31 men, 21 women; mean age 27.8±5.0 yr; range 18-39 yr) and 20 healthy volunteers (12 men, 8 women, mean age 26.4±5.3 yr; range 19-38 yr) were included in this study. The protocol followed the tenets of the Declaration of Helsinki. All patients had complete ophthalmologic and systemic examination to define ophthalmic and systemic involvement and disease activity.

The diagnostic criteria for Behçet’s Syndrome proposed by the International Study Group for Behçet’s Disease were used for diagnosis [23]. These criteria include a required element of recurrent oral ulceration, plus at least two of the following: recurrent genital ulceration, ocular inflammation, skin lesions, and positive pathergy test (ie, local inflammatory reaction to scratches or intradermal saline injection).

BD cases were assigned to 3 groups according to the activity of uveitis and other findings of BD disease. The patients all had oral ulcerations, genital ulcerations, and positive pathergy test either previously or concurrently with the study. If ocular findings such as anterior uveitis, posterior uveitis, cells in vitreous humor on slitlamp examination, or retinal vasculitis were present, the patients were assigned to the “active ocular BD group.” If the patients with ocular findings mentioned above were treated and showed no activity during the past 6 mo, they were assigned to the “inactive ocular BD group.” The “nonocular BD group” consisted of patients without ocular findings at any time. The controls included 20 sex- and age-matched healthy volunteers without systemic or ocular disease.

Erythrocyte sedimentation rate (ESR) was determined by the Westergren method using anticoagulated whole blood. Venous blood was collected in Vacutainers without additives, allowed to clot 30 min at room temperature, and centrifuged at 3000 x g for 5 min to separate serum. Serum samples were stored at -80°C. Hemolysed samples were excluded.

Serum MDA was determined by the thiobarbituric acid method [24]. Serum aliquots (0.2 ml) were mixed thoroughly with 0.8 ml of phosphate-buffered saline (pH 7.4) and 0.025 ml of butylated hydroxytoluene solution. After addition of 0.5 ml of 30% trichloroacetic acid, the samples were placed on ice for 2 hr and then centrifuged at 3000 x g for 5 min to separate serum. Serum samples were stored at -80°C. Hemolysed samples were excluded.

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MDA, using a molar extinction coefficient for MDA of $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$. The results were expressed as nmol/dl.

CuZn-SOD activity was assayed according to Sun et al [25]. In this method, a xanthine-xanthine oxidase complex produces superoxide radicals, which react with nitroblue tetrazolium (NBT) to form the formazone compound. The SOD activity is measured at 560 nm by detecting the inhibition of this reaction. One unit of SOD activity is defined as the activity that causes 50% inhibition of NBTH$_2$ reduction rate. SOD activity was expressed as U/dl.

GSH-Px activity was assayed according to Paglia and Valentine [26]. In this method, GSH-Px catalyzes the oxidation of glutathione in the presence of tert-butyl hydroperoxide. Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to NADP. The reduction in absorbance of NADPH at 340 nm is measured. The absorbance change per min and the molar extinction coefficient of NADPH are used to calculate GSH-Px activity. GSH-Px activity was expressed as IU/dl.

Serum Cp, Alb, Trf, and C-reactive protein (CRP) levels were determined by nephelometry using the Beckman Array 360 Protein System (Beckman Instruments, Brea, CA).

For statistical analysis, parameters were analyzed by one-way analysis of variance (ANOVA). The least significant difference (LSD) multiple range test was used to compare means (significance = $p < 0.05$). Correlations between variables were determined by Pearson’s rank correlation analysis. Statistical analyses were performed with the Statistical Package for the Social Sciences (version 10.0, SPSS, Inc., Chicago, IL). Results are reported as mean ± SD.

### Results

The results for all parameters are listed in Table 1. Of 52 BD patients, 19 were active ocular BD, 18 inactive ocular BD, and 7 nonocular BD. There were no statistically significant differences between the groups when either age or sex were considered.

Serum MDA levels were higher in patients with active ocular BD, inactive ocular BD, and nonocular

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<table>
<thead>
<tr>
<th>Table 1. Results (mean ± SD) of biochemical assays in the patients with Behçet’s disease (BD) and control subjects.</th>
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<tbody>
<tr>
<td><strong>Active</strong></td>
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<td><strong>ocular BD</strong></td>
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<tr>
<td><strong>number of subjects</strong></td>
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<td><strong>male</strong></td>
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<td><strong>female</strong></td>
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<td><strong>age (yr)</strong></td>
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<td>serum malondialdehyde (MDA) (nmol/dl)</td>
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<td>serum CuZn-SOD (U/dl)</td>
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<td>serum GSH-Px (IU/dl)</td>
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<td>serum albumin (Alb) (g/L)</td>
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<td>serum ceruloplasmin (Cp) (mg/dl)</td>
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<td>serum transferrin (Trf) (mg/dl)</td>
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<td>erythrocyte sedimentation rate (ESR) (mm/hr)</td>
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<td>serum C-reactive protein (CRP) (mg/L)</td>
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$^a p < 0.05$, $^b p < 0.01$, $^c p < 0.001$, compared to the control group.
$d p < 0.05$, $^e p < 0.01$, $^f p < 0.001$, compared to the inactive ocular BD group.
BD, compared with the control group. Serum MDA levels in patients with active ocular and nonocular BD were significantly higher than in the inactive ocular BD group.

Serum CuZn-SOD activities in all patient groups were significantly higher than the control group. The active ocular BD group had significantly higher serum CuZn-SOD activities than the inactive ocular BD group. Serum GSH-Px activities were higher in patients with active ocular BD, inactive ocular BD, and nonocular BD, compared with the control group.

Serum Cp levels were higher in patients with active ocular BD, inactive ocular BD, and nonocular BD, compared with the control group. Serum Trf levels were lower in patients with active ocular BD, inactive ocular BD, and nonocular BD, compared to the control group.

Blood ESR and serum CRP levels were higher in patients with active ocular BD, inactive ocular BD, and nonocular BD compared with the control group. The active ocular BD and nonocular BD groups also had significantly higher blood ESR and serum CRP levels compared to the inactive ocular BD group.

No meaningful differences were found in serum albumin levels in the groups of patients and controls. There was significant positive correlation between serum CP and Trf levels in the controls (r=0.44, p <0.05). Serum MDA showed significant positive correlation with blood ESR (r=0.48, p <0.01) and CRP levels (r=0.52; p <0.01) in patients with active ocular BD and in patients with nonocular BD (r=0.66, p <0.01; r=0.83, p <0.001, respectively). No significant correlations were found among the other parameters in healthy controls and BD patients.

Discussion

The etiology and pathogenesis of Behçet’s disease are unknown. Bacterial and viral infections have been considered as causes, but no convincing evidence exists for these suggestions. Since the disease is characterized by vasculitis, the underlying mechanism may be an autoimmune process [17]. Abnormalities of neutrophil functions, such as chemotaxis, phagocytosis, and especially ROS generation (including the superoxide radical anion, O₂⁻) have been proposed as factors in the etiology and pathophysiology of BD. It has been suggested that the vascular and endothelial tissue damage in BD may reflect enhanced production of ROS by activated neutrophils [12,27].

As is well known, ROS produced in excess may cause toxic effects by oxidative damage of molecules, membranes, and tissues. Above all, the oxidation of membrane lipids has been implicated as one of the primary events in oxidative cellular damage. The most common approach to determine the degree of lipid peroxidation induced by ROS is to measure breakdown products such as MDA [28,29].

Lipid peroxidation in serum of patients with BD was increased in agreement with previous reports [11,12,27,30]. This confirms the presence of increased oxidative stress in BD. Decreased sulfhydryl levels have been reported in serum of patients with BD [12]. The positive correlation that was observed in this study between the serum MDA level and blood ESR and between the serum MDA and CRP levels in patients with active ocular BD and nonocular BD are consistent with a cause-and-effect relationship (ie, the higher the oxidative stress, the worse the clinical condition of the patient).

Increased CuZn-SOD and GSH-Px activities were found in the serum of patients with BD in the present study. Contrary to this, decreased SOD and unchanged GSH-Px activity have been reported in erythrocytes of patients with BD [31]. Kose et al [12] found decreased GSH-Px activity in serum of BD patients. The reasons for these discrepancies remain to be clarified. SOD dismutates the superoxide radical (O₂⁻) to H₂O₂ [32]. GSH-Px detoxifies H₂O₂ by converting it to water and molecular oxygen [33]. H₂O₂ is not a free radical; however, it can be converted to hypochlorous acid (HOCl) and hydroxyl radical (HO·) by myeloperoxidase in neutrophils [34]. These reactive radicals might be responsible for the oxidative damage reflected as increased serum MDA levels in patients with BD in our study.

Increased Cp levels were increased and serum Trf levels were decreased in patients with BD in this study. These data confirm an earlier study [12]. Increased [35,36] serum Cp levels, but unchanged
Recent studies have reported increased serum transferrin (Trf) levels in patients with various autoimmune diseases. In line with these findings, increased serum ceruloplasmin (Cp) and decreased Trf levels in patients with rheumatoid arthritis, have recently been reported. Of the extracellular antioxidants, ceruloplasmin oxidizes Fe^{2+} to Fe^{3+} and facilitates the binding of Fe^{3+} to Trf. Trf inhibits iron-ion dependent HO· formation from H_{2}O_{2}. Iron-catalyzed reactions are limited by the presence of Trf in human plasma. The increased Cp level found in BD patients may be a protective response to an increase in circulating unbound Fe^{2+}, which may act as a catalyst for free-radical induced lipid peroxidation. Positive correlation between Cp and TF in the healthy control subjects may suggest collaboration between these two extracellular antioxidants. However, such collaboration seems to be impaired in patients with BD. On the other hand, decreased Trf levels may have led to iron-dependent HO· formation from H_{2}O_{2} in these patients. Supporting this proposed mechanism involving hydroxyl radicals in BD, Shingu et al. have shown that addition of free metal ions, Fe^{2+} or Cu^{2+}, to sera from patients with systemic lupus erythematosus promoted H_{2}O_{2}-mediated complement activation; normal serum cultured with fibroblasts for 24 hr showed complement activation via a catalase-inhibitable process.

In conclusion, in patients with BD, increased O_{2·} radicals could be dismutated to produce H_{2}O_{2} by increased serum CuZn-SOD activity, and the H_{2}O_{2} could be converted to more reactive free radicals, such as hydroxyl radical (HO·), by iron owing to diminished serum Trf levels. These factors may contribute to the oxidative damage reflected as increased serum MDA levels. Our results confirm the presence of oxidative stress in patients with BD and show the need for further studies on this subject.

References


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