

Oxidant/Antioxidant Status in Patients with Behçet Disease

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Abstract

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Background. Increased oxidant stress and decreased antioxidant enzyme activities may result in tissue injury in Behçet's disease (BD) patients.

Aim. The aim of the study was to assess various selected markers of oxidative stress in patients with BD.

Material and Methods. Thirty-two patients with BD and twenty-five healthy age- and sex- matched controls were recruited from the Rheumatology Department of Cairo University Hospital. Behçet's Disease current activity form (BDCAF) was used in order to assess the disease activity. The following parameters were assayed: serum nitric oxide level (NO), serum malondialdehyde (MDA), serum hydrogen peroxide (H₂O₂), serum total antioxidant capacity (TAC).

Results. The present result showed that serum NO and TAC levels were significantly lower in BD patients, than in healthy controls (P<0.0001). Serum MDA and H₂O₂ were significantly higher in BD patients than in healthy controls (P<0.003 and P<0.001 respectively).

Conclusion. Our results confirm the presence of oxidative stress in patients with Behçet's Disease and suggest that the severity of Behçet's Disease may arise from impaired antioxidant mechanisms.

Introduction

Behçet's disease (BD), a chronic inflammatory disease, is a multisystem disorder characterized by recurrent oral ulcerations, genital ulcerations, inflammation of the eye and skin lesions. It can also involve musculoskeletal, gastrointestinal tract and central nervous system. Vasculitis of small and large blood vessels is the main histopathology in BD [1]. The etiology of this disease is still unknown, but the most accepted hypothesis is that an inflammatory response triggered by an infectious agent

in a genetically susceptible host can cause the disease [1].

Free radicals act as signaling species in various normal physiological processes. However, excessive production of free radicals causes damage to biological materials and is an essential event in the etiopathogenesis of various diseases [2]. Furthermore, lipid peroxidation has invariably been found to be accelerated in both plasma and erythrocytes of patients with BD and is characterized by increased malondialdehyde (MDA) levels or in vitro low

density lipoprotein oxidation [3]. Measurement of oxidative stress factors such as MDA and nitric oxide (NO) and accompanying evaluation of the antioxidant defense system can be significant in the diagnosis, treatment and follow up of BD [4].

The present work was carried out to assess various selected markers of oxidative stress and to determine whether they can be used as a diagnostic or activity marker in BD patients.

Design and Methods

Patients included in this study were selected from attendants of Rheumatology and Rehabilitation outpatient clinic and inpatient department of Cairo University Hospital. 57 participants were included in this study; thirty two patients with BD not receiving specific diet, (30 males and 2 females) aged 22-50 years (mean age 35.03 ± 7.7 years) and twenty five healthy volunteers (23 males and 2 females) aged 25-47 years (mean age 33.1 ± 5.5 years) served as control group.

The selection of our patients was based on the criteria of the International Study Group (ISG) [5] for the diagnosis of BD. Behçet's Disease Current Activity Form (BDCAF) was used to assess the disease activity of these patients [6]. All patients were subjected to detailed medical history and thorough clinical examination including skin and ocular examination. Pathergy test was performed for all patients by intradermal saline injection under the forearm skin with a 20-gauge needle, 5 mm deep obliquely. Pathergy test is considered positive if a sterile pustule of 3–10 mm was observed after 24–48 h at the needle prick site in the skin. The patients with dyslipidemia, cardiovascular diseases, diabetes mellitus, renal failure, chronic infections and those who used antioxidant or systemic drugs for at least 3 months before blood sampling were excluded from the study.

All patients gave informed consent and the study was approved by the Institutional Ethical Committee.

Sample Collection and Storage

Venous blood sample (10 mL) was withdrawn from each participant after an overnight fasting and divided into two parts:

- Five mL of the sample was taken in EDTA containing tube for determination of complete blood count (CBC) using automated blood counter (Coulter T660) and erythrocyte sedimentation rate (ESR) estimated by

classical Westergren method [7]

- The rest of the sample was collected in plain tube free from anticoagulant, allowed to clot for 30 min at room temperature, then was centrifuged at 2000 xg for 15 min at 4°C. Serum was separated, divided into aliquots and stored at -80°C until assayed within one month.

Biochemical Parameters

Serum samples were used for determination of the following parameters:

- C-reactive protein (CRP) was measured using a Cobas 400 chemical analyzer system [8].
- Liver function tests were performed using a Beckman Auto-analyzer (Synchron CX4, USA) [9].
- Kidney function tests were measured using Auto-analyzer techniques, colorimetric techniques were used for the determination of blood urea by Berthelot's reaction [10] and serum creatinine was determined by alkaline picrate method [11].
- Serum malondialdehyde (MDA) level was colorimetrically determined using a kit provided by Bio-diagnostic Company Egypt. Thiobarbituric acid reacts with MDA in acidic medium at temperature of 95°C to form thiobarbituric acid reactive product. The absorbance of the resultant colored product can be measured spectrophotometrically at a wavelength 534nm concentration [12]. Linearity was up to 100 nmol/mL.
- Serum hydrogen peroxide (H_2O_2) level was colorimetrically determined using a kit provided by Bio-diagnostic Company Egypt. In the presence of peroxidase, hydrogen peroxide reacts with 3, 5-dichloro-2-hydroxy benzenesulfonic acid and 4-amino antipyrine peroxidase. The resultant color can be measured spectrophotometrically at 510nm [13]. Linearity was up to 1.5 mM/L.
- Serum total antioxidant capacity (TAC) level was colorimetrically determined using a kit provided by Bio-diagnostic Company Egypt. In this procedure the determination of the antioxidative capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously- provided hydrogen peroxide. The antioxidants in the sample eliminate certain amount of the provided hydrogen peroxide. The residual hydrogen peroxide amount is determined colorimetrically (500-510 nm) by an enzymatic reaction which involves conversion of 3, 5-dichloro-2-hydroxy

benzenesulphonate to a colored product [14]. Linearity up to 2mM/L.

Serum level of nitric oxide (NO) was accomplished using quantitative Total NO/Nitrite/Nitrate kit manufactured by R&D Systems, USA, (Catalog Number KGE001). Serum samples were ultra filtered through 10,000 MW filters and were diluted a 2-fold dilution prior to assay. Endogenous nitrite is measured firstly. Then, the total nitrite is measured as nitrate is converted to nitrite using nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction [15]. The nitrate concentration is determined by subtracting the endogenous nitrite concentration from the total nitrite concentration. Since samples have been diluted, the concentration read from the standard curve was multiplied by the dilution factor. Intra-assay and Inter-assay variations were 1.4% and 3.6% respectively.

Statistical Analysis

Data were statistically described in terms of range; mean ± standard deviation (± SD). Comparison of quantitative variables between different groups was done by Mann Whitney U test. Chi square (c²) test and Pearson correlation between various variables were evaluated. A probability value (p value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA), version 13, statistical program.

Results

Thirty-two patients with BD; diagnosed according to ISG criteria for BD, thirty males and two females, with BDCAF score ranging from zero and 13 (mean ± SD: 5.19

Table 1: Demographic and laboratory characteristics of BD patients and control group.

| | Patients | Control |
|----------------------------------|------------------|--------------|
| Age (years) | 35.03 ± 7.7 | 33.1 ± 5.5 |
| Age of onset (years) | 27.9 ± 7.62 | -- |
| Disease duration (months) | 86.03 ± 68 | -- |
| Hb % | 4.9 ± 0.3 | 5.4 ± 1.1 |
| TLC (10 ³ /cmm) | 7.856 ± 1.832 | 8.52 ± 1.4 |
| Platelets (10 ³ /cmm) | 278.178 ± 70.782 | 295 ± 82.512 |
| Creatinine (mg/dl) | 0.85 ± 0.18 | 0.4 ± 0.2 |
| Albumin (g/dl) | 3.9 ± 0.49 | 4.1 ± 0.1 |
| ALT (U/L) | 34.7 ± 25.41 | 24.1 ± 4.3 |
| AST (U/L) | 29.7 ± 10.25 | 19.4 ± 9.3 |
| ESR (mm/hour) | 13.84 ± 8.3 | 12.5 ± 5.3 |

Hb: hemoglobin; TLC: total leucocytic count; ALT: alanine transaminase; AST: aspartate transaminase; ESR: erythrocyte sedimentation rate.

± 3.2) and 25 healthy volunteers (23 males and 2 females) aged 25-47 years (mean age 33.1 ± 5.5 years) served as control group. Demographic and laboratory characteristics of the patients and controls are presented in Table 1.

Markers of oxidative stress in BD patients and in controls are presented in Table 2. We have detected higher serum levels of MDA and H₂O₂ in BD patients compared to controls (p=0.003 and p<0.001 respectively). TAC and NO were significantly lower in BD patients compared to controls (p<0.001).

Table 2: Markers of oxidative stress in control group and BD patients.

| Parameter | Control group | BD group | p value |
|--|---------------|-------------|---------|
| MDA (nmol/ml) | 2.82 ± 0.5 | 3.35 ± 0.69 | 0.003 |
| H ₂ O ₂ (mmol/L) | 2.5 ± 0.71 | 3.7 ± 0.97 | <0.001 |
| TAC (mmol/L) | 1.4 ± 0.33 | 0.94 ± 0.21 | <0.001 |
| NO (mol/L) | 39.36 ± 12.67 | 28 ± 11.53 | 0.0001 |

Data presented as mean ± SD; MDA: malondialdehyde; H₂O₂: hydrogen peroxide; TAC: total antioxidant capacity; NO: nitric oxide.

Table 3 shows the correlation between the studied markers of oxidative stress and the BDCAF score of the BD patients, there was no significant correlation between BDCAF scores and H₂O₂, MDA, TAC, or NO.

Table 3: Correlation between markers of oxidative stress and the BDCAF score in BD patients.

| Studied parameter | r | p value |
|--|--------|---------|
| MDA (nmol/ml) | 0.230 | 0.204 |
| H ₂ O ₂ (mmol/L) | 0.269 | 0.137 |
| TAC (mmol/L) | -0.074 | 0.689 |
| NO (mol/L) | 0.144 | 0.431 |

MDA: malondialdehyde; H₂O₂: hydrogen peroxide; TAC: total antioxidant capacity; NO: nitric oxide.

Table 4 shows the correlation between the studied markers of oxidative stress (MDA, H₂O₂, TAC, NO) and the demographic characteristics of the BD patients.

Table 4: Correlation between the oxidative stress parameters (MDA, H₂O₂, TAC, NO) and the demographic characteristics of BD patients.

| Patients (n=32) | MDA (nmol/ml) | | H ₂ O ₂ (mmol/L) | | TAC (mmol/L) | | NO (mol/L) | |
|------------------|---------------|----|--|----|--------------|----|------------|----|
| | r | p | r | p | r | p | r | p |
| Age | -0.06 | NS | -0.30 | NS | 0.12 | NS | 0.01 | NS |
| Age of onset | -0.14 | NS | -0.26 | NS | -0.02 | NS | -0.14 | NS |
| Disease duration | 0.12 | NS | -0.05 | NS | 0.20 | NS | 0.20 | NS |
| BMI | -0.08 | NS | -0.07 | NS | -0.06 | NS | -0.20 | NS |

NS = Non-significant.

We studied the correlation between markers of oxidative stress with each other, we found only a significant positive correlation between MDA and H₂O₂ (Table 5).

Table 5: Correlation between the oxidative stress markers with each other.

| Patients (n=32) | MDA (nmol/ml) | | H ₂ O ₂ (mmol/L) | | TAC (mmol/L) | | NO (mol/L) | |
|--------------------|------------------|----|---|----|-----------------|----|---------------|----|
| | r | p | r | p | r | p | r | p |
| Age | -0.06 | NS | -0.30 | NS | 0.12 | NS | 0.01 | NS |
| Age of onset | -0.14 | NS | -0.26 | NS | -0.02 | NS | -0.14 | NS |
| Disease duration | 0.12 | NS | -0.05 | NS | 0.20 | NS | 0.20 | NS |
| BMI | -0.08 | NS | -0.07 | NS | -0.06 | NS | -0.20 | NS |

Discussion

Behçet's disease is a multisystem disorder that affects eyes, mucous membranes, skin, joints, central nervous system and blood vessels. The disease is prevalent in Middle Eastern and East Asian countries [16]. Growing evidence indicates that oxidative stress is increased in BD, owing to the overproduction of reactive oxygen species and decreased efficiency of antioxidant defenses [17].

In the present study, serum levels of H₂O₂ and MDA were significantly higher in BD patients than in controls. Elevated serum MDA levels in BD patients have been reported by many investigators [16, 18-22]. In BD an excessive amount of superoxide was produced in active polymorphic nuclear leucocytes, and plasma MDA levels were elevated as a result of lipid peroxidation caused by the superoxide radicals [18]. Increased MDA levels confirm the presence of increased oxidative stress in BD patients [16].

Hydrogen peroxide (H₂O₂) was significantly higher in our patients than in controls. Hydrogen peroxide is toxic to cells and is a cause of further free radical generation that is implicated in the pathogenesis of a variety of diseases [23]. Hydrogen peroxide per se is not a free radical; however, it can be converted to hypochlorous acid and hydroxyl radical by myeloperoxidase enzyme present in phagocytes. These more reactive radicals may lead to oxidative damage and increased H₂O₂ may lead to oxidative damage reflected as increased MDA levels [16]. This may explain the significant positive correlation between H₂O₂ and MDA in our study.

Serum levels of NO were lower in our patients compared to controls. Nitric oxide is a powerful vasodilator; it is produced from endothelial nitric oxide synthase (NOS). Decreased activity of endothelial NO leads to vasoconstriction, platelet aggregation and monocyte adhesion and this may explain increased risk of thrombosis in BD patients. Previous studies reported lower NO levels in active BD compared to inactive BD patients and healthy controls [18,24,25], Other investigators reported increased NO in active BD compared to inactive BD [20,26,27].

These contradictory results may be due to genetic variation as NOS polymorphism may affect transcription or function of NO. Also NO may be affected by diet, hydration and renal function [19]. Our study didn't show significant correlation between BDCAF scores and NO levels in BD patients. This may be explained by using different indices in determining the disease activity state or due to the low statistical power limited by sample size.

Total antioxidant capacity (TAC) which is an indicator of the overall function of enzymatic and non enzymatic antioxidant molecules in the serum was found to be lower in BD patients compared to healthy controls which indicate that antioxidant defense system is deficient or inadequate in BD patients [28, 29]. However, Bekpınar and colleagues (2005) [19] reported no significant difference in TAC between BD patients and controls.

Insignificant correlation was found between serum levels of the markers of oxidation (MDA, H₂O₂, TAC and NO) measured in this study, and the age, age of onset, disease duration or even BMI among the studied BD patients. Kural-Seyahi and colleagues (2003) [30] reported that BD is more severe in young males, and that the severity and the mortality tend to decrease significantly with the passage of time.

In the present study, there was insignificant correlation between BDCAF scores and MDA, H₂O₂ and NO. Some authors supported our results [18, 25]; while others contradicted [19, 20] our result.

In concordance with our results, others suggested that chlorinated oxidants of neutrophil origin may be the major source of oxidative stress in BD resulting mainly in protein oxidation, and that the lipid peroxidation may not be the major source of oxidative end products in BD. They even concluded that advanced oxidation protein products (AOPP) may be more useful marker than MDA in monitoring the activity of BD [3].

Conclusion

Behçet's disease patients are subjected to oxidative damage reflected by increased serum MDA and H₂O₂ and decreased serum TAC, however these oxidative markers are not related to the activity of the disease.

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