

ORIGINAL ARTICLES

Oxidative stress markers in patients with Behcet's disease

Trifa A. Abdulghafur Rahimi¹, Mohammed Tahir Rasool²¹Directorate of Health-Erbil, Erbil, Kurdistan Region, Iraq²Department of Medicine, Faculty of Medical Sciences, University of Duhok, Duhok, Kurdistan Region, Iraq**Abstract**

Aim: To evaluate the role of serum malondialdehyde (MDA) and nitric oxide (NO) as oxidative biomarkers in patients with Behcet's disease (BD)

Methods: The case-control study included 44 cases and 44 controls from different areas of Duhok governorate, Iraq. All the subjects had undergone serological and hematological examinations.

Results: The male to female ratio noted was 1.1:1, and the age of patients ranged between 10-63 years. The mean age of the subjects was 37 years at presentation. Serum MDA level, ESR, and total number of granulocytes were significantly higher in patients with BD in comparison to healthy controls. Furthermore, mean serum NO and the number of lymphocytes were significantly lower in BD patients compared to controls. However, total white blood cell count and average number of monocytes were comparable in both the groups.

Conclusion: The study indicated the role of MDA and NO in the pathogenesis and progression of Behcet's disease. Further research is warranted to explore its implication in diagnosis and treatment of BD.

Keywords: Malondialdehyde, Nitric Oxide, Oxidative stress, Behcet's Disease

Introduction

In 1937, Hülusi Behçet (1889-1948), the Turkish dermatologist comprehensively described the symptom complex of recurrent oral aphthous ulcers, genital ulcers, and uveitis as a disease entity. He also suggested the possible viral etiology.¹ Nowadays, Behcet's disease (BD) has been identified as an inflammatory disorder characterized by recurrent oral aphthous ulcers and at least two of the following features: recurrent genital ulcers, eye involvement, skin lesions, and a positive pathergy test.² Although the etiology of BD is obscure, it is believed to be triggered by environmental factors such as microbial agents in individuals with a particular genetic background and their interactions.³ The underlying histopathologic feature is systemic vasculitis affecting veins, arteries, and capillaries.⁴

Malondialdehyde is a breakdown product of lipid

peroxidation and it is the most commonly used, validated index of oxidative stress.⁵ It is directly formed by β -scission of a 3-hydroperoxyaldehyde or by reaction between acrolein and hydroxyl radicals.⁶ Serum MDA levels have been reported to be higher in patients with active and inactive ocular BD, and non-ocular BD compared to the control group.⁷ Studies have also found increased MDA levels in patients with active disease than those inactive disease.⁸ Furthermore, highly elevated serum MDA in patients with BD is an evidence of increased lipid peroxidation during the active stage of the disease.⁹ It has been described that reactive oxygen species (ROS) can attack double bonds in polyunsaturated fatty acids and induce lipid peroxidation, which in turn results in further oxidative damage. ROS-mediated oxidation of cell membrane lipids leads to the formation of lipid peroxidation products, such as MDA. A similar mechanism was described by Kose *et al.* (2001).¹⁰ The researchers have found that increased plasma

MDA levels in BD patients are caused by excessive ROS production by activated neutrophils and the presence of an impaired oxidant/antioxidant balance in both neutrophils and plasma.

NO, synthesized by endothelial cells, is an important molecule for the vascular system. It plays a key role as a mediator of immunity and inflammation, and its functions include the inhibition of platelet adhesion and endothelial vasodilatation.¹¹ The synthesis of NO is simple and involves one of the most complicated enzymes in nature, the NO synthase (NOS). The synthesis needs several co-factors and is highly regulated.¹² L-arginine, the substrate for NO synthesis, undergoes an electron oxidation to form L-citrulline and the free radical NO.¹³ A reduction in NO levels has been reported in patients with active BD, and this may have a critical role in the development of the endothelial abnormalities and thrombotic tendency observed in BD patients.¹⁴ It is plausible that increased free radical production through oxidative stress might be involved in events leading to suppression of NOS activity in endothelial cells.¹⁵ On the other hand, Arayssiand and Hamdan (2004) have described that the dysfunction of NOS leads to reduced NO levels in patients with BD, particularly in those with active disease.¹⁶

There are contradictory findings on the mechanism of pathological progression of BD. As per the literature review, there is no study from Duhok governorate, Iraq elucidating the role of MDA and NO in BD. The present study is the first of its kind evaluating serum NO and MDA as oxidative biomarkers in patients with BD from this governorate. It has also studied the role of inflammatory markers such as erythrocyte sedimentation rate (ESR) and white blood cells in the diagnosis of patients with BD.

Materials and methods

The present case-control study included patients who attended the Duhok Center of Rheumatic Diseases and Rehabilitation for non-specific mechanical strain or sprain and with no history of orogenital ulceration, and 46 subjects who served as healthy controls. The study was approved by Medical Ethics Committee, Faculty of Medical Sciences, University of Duhok, Iraq. Data were collected from all age- and sex- matched subjects, and verbal concepts of agreement were taken for their involvement in the study. The inclusion criteria considered were history of recurrent orogenital ulceration on three or more occasions for more than one year and the presence of two of the clinical manifestations adopted by International Study Group (ISG)

criteria for the diagnosis of BD.

Blood samples (5ml) drawn from a peripheral vein in the morning (8:30-10:30AM), avoiding hemolysis, were collected in plain tubes. The samples were divided into two tubes with or without anticoagulant (ethylenediaminetetraacetic acid), the first half of the blood samples with anticoagulant was used for hematological investigations. The other half of the blood samples were centrifuged at 3500 rpm for 10 min to obtain serum, and then collected samples were kept at -80°C until analysis. Total white blood cells (WBCs) and differential leukocyte counts (DLC) were determined using automated hematology analyzer. ESR in the first hour was determined by Westergren method (Chawla, 2003).

Serum MDA levels were measured according to a method described by Karakucuk *et al.* (2004).⁹ The principle of the method is based on the spectrophotometric measurement of the colour occurring during the reaction of thiobarbituric acid (TBA) with MDA. The concentration of thiobarbituric acid reactive substances, expressed as nmol/ml, was calculated by the absorbance coefficient of the malondialdehyde-thiobarbituric acid complex. MDA bis (dimethyl acetal)-TBA complex was used as standard for the assessment.

Nitric oxide was determined by Griess reaction. In this method, nitrite is first treated with a diazotizing reagent, e.g. sulfanilamide, in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED) to form a stable azo compound. The intense purple colour of the product allows nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as ~0.5 mM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample.¹⁷

Data were presented as mean±standard error of the mean. The significance of the mean differences between control and BD groups was assessed by unpaired Student's t-test using GraphPad Prism 5 (GraphPad Software, USA). P value <0.05 was considered to be statistically significant.

Results

The study recruited 44 patients and the male to female ratio noted was 1.1:1 (23 male and 21 female). The age of the selected subjects ranged between 10-63 years, and the mean age noted was 37 years at presentation. Most of the subjects belonged to the age group of 20-39 years

(59.1%, Table 1).

Out of the 44 selected subjects, 12 (27.3%) had positive family history of BD. The major clinical manifestations of BD noted were oral ulcer, genital ulcer, skin lesions and eye lesions. The most frequent manifestation noted was oral ulcer (occurred in all cases,100%), followed by skin

lesions (70.5%), genital ulcer and eye lesions (61.4%), and cardiovascular system manifestations (13.6%). The frequency of central nervous system, gastrointestinal system and musculoskeletal system manifestations noted were 40.9%, 31.8% and 68.2% respectively (Table 2).

The mean level of ESR in BD patients was found to be

Table 1: Distribution of age and gender in patients with BD

Year	Gender		Total	
	Female	Male	Number	Percentage
Below 20	1	1	2	4.5
20-39	13	13	26	59.1
Over 40	7	9	16	36.4
Total	21	23	44	100

Table 2: Family history and clinical manifestations in BD patients

Clinical manifestations	Number	Percentage
Family history	12	27.3
Oral ulcer	44	100
Genital ulcer	27	61.4
Skin lesions	31	70.5
Eye lesions	27	61.4
Central nervous system	18	40.9
Cardiovascular system	6	13.6
Gastrointestinal system	14	31.8
Musculoskeletal system	30	68.2

Table 3: The level of MDA, NO and ESR in patients with Behçet's disease and the control subjects

Parameters	Control group*	BD group*	P value
MDA ($\mu\text{mol/L}$)	4.34 \pm 0.14	11.08 \pm 0.67	<0.001
NO ($\mu\text{mol/L}$)	388.3 \pm 37.01	107.1 \pm 8.75	<0.001
ESR (mm/hr)	14.52 \pm 2.36	24.18 \pm 2.78	<0.05

*Mean \pm SD

Table 4: Total white blood cells, monocyte, lymphocyte and granulocyte count in the patients with Behçet's disease and the control subjects

Parameters	Control group*	BD group*	P value
Total white blood cells ($10^9/\text{L}$)	7.37 \pm 0.26	7.92 \pm 0.36	NS
Monocyte ($10^9/\text{L}$)	0.47 \pm 0.03	0.46 \pm 0.02	NS
Lymphocyte ($10^9/\text{L}$)	2.38 \pm 0.11	2.01 \pm 0.10	<0.05
Granulocyte ($10^9/\text{L}$)	4.38 \pm 0.18	5.40 \pm 0.34	<0.01

*Mean \pm SD, NS: non-significant

significantly higher than controls. Furthermore, the mean serum value of MDA was significantly elevated in patients with BD in comparison to controls. On the other hand, mean serum NO was significantly decreased in BD patients compared to controls (Table 3).

Total white blood cell and monocyte counts were similar in both BD patients and control groups. On the other hand, the number of lymphocytes was significantly decreased in patients with BD compared to controls. While, the total number of granulocytes was statistically increased in patients with BD compared to controls (Table 4).

Discussion

BD is commonly noted in patients belonging to their third and fourth decades of life. In the present study, the age of presentation noted was between 20-39 years and the mean age of distribution was 37 years. Age of onset of BD noted in different countries namely Iran, Japan, China, Germany, Turkey, Korea, and United Kingdom was 26.1, 35.7, 33.8, 26, 25.6, 29, and 24.7 respectively.¹⁸ The gender distribution has also been found to be varying across countries. The male-to-female ratio noted in the present study was 1.1:1. A similar ratio was observed in Duhok province and a little bit higher ratio (1.16) in Erbil city.^{19, 20} In another study, the ratio noted in Jordan was 2:1.²¹ The ratio noted in different countries was as follows: 0.98 in Japan, 0.63 in Korea, 1.19 in Iran, 1.03 in Turkey, 1.8 in India, 3.4 in Saudi Arabia, 4.9 in Kuwait, 3 in Iraq, 2.8 in Jordan, 1.3 in Lebanon, and 2 in Morocco.²²

Among the 44 BD patients participated in the current study, 13 of them had positive family history (27.3%). Both genetic and environmental factors contribute to the development of the disease. BD is more common among females in Japan and Korea, whereas it is prevalent among males in the Middle Eastern countries. The frequency noted within families was 2% to 5%, except in Middle Eastern countries (10% to 15%).²³

Familial aggregation has been considered as the evidence supporting the genetic predisposition to BD, mostly in patients of Turkish, Israeli or Korean origin. Increased predisposition has also been observed in patients with juvenile disease and in those belonging to the families of probands carrying HLA-B51. Variable frequencies of familial aggregation of BD noted in different ethnic groups were as follows: Turks (18.2%), Koreans (15.4%), Jews (13.2%), Chinese (2.6%), Japanese (2.2%), and Europeans (1%).³

Major clinical manifestations of BD noted in the current study include oral ulcer, genital ulcer, skin lesions and eye lesions. Oral ulcer (100%) was the most frequent manifestations noted, followed by skin lesions (70.5%), and genital ulcer and eye lesions (61.4%). These findings are in agreement with the study by Kump *et al.* (2008) who concluded that all the patients had recurrent oral ulcers.²⁴ Genital ulcers were the second most common sign (69%), followed by skin lesions (58%), arthritis (38%), central nervous system manifestations (16%), vascular lesions (5%), and epididymitis (4%).

Oral aphthosis (OA) is the most frequent and common manifestation noted in BD patients. The average prevalence noted from the eight large studies, conducted in Iran, Japan, Turkey, China, Korea, Morocco, Germany, and United Kingdom, was 98%.¹⁸ Skin lesions occur in about 80% of patients and can be divided into two main types namely, erythema nodosum and papulopustular/acneiform lesions. The erythema nodosum-like lesions, occurring commonly on the legs, do not ulcerate and produce hyperpigmented areas on healing.²⁵ Literature evidence indicate a wide variation in the prevalence of skin manifestations of BD, localized mainly in the lower limbs (range 39% to 93%).²⁶

Ocular diseases, causing symptoms such as blurring of vision, eye pain, photophobia, tearing, and floaters, occur in 25% to 75% of the patients. The diseases include recurrent anterior uveitis with or without hypopyon, posterior uveitis and retinal vasculitis.²⁷ The percentages of ocular manifestations reported in Iran, Japan, China, Korea and Germany (referred to as the five nation studies) were 55%, 69%, 35%, 51% and 55% respectively. A high prevalence of eye involvement has also been reported in Arab countries such as Morocco (67%) and Jordan (60%).²¹

Genital ulcers, occurring in 60%–80% of cases, are less common than oral ulcers. They can be painful leading to difficulty in sitting down and walking, pain on intercourse, and dysuria. Morphologically, they are similar to mouth ulcers and their appearance can be preceded by a tender nodule. The scrotum is the most frequently involved site in males, ulcers on the shaft and glans penis are also reported. The ulcers most commonly occur on the labia in females and they may occur in vagina and cervix also.²⁵

The corresponding percentages of musculoskeletal, central nervous, gastrointestinal and cardiovascular system disorders reported in the present study were 68.2%, 40.9%, 31.8% and 13.6%. The joint manifestations

of BD were found in 16% to 93% of the patients.²⁸ Joint involvement was observed in half of the patients in the present study. Arthritis is usually non-deforming and non-erosive, mono- or oligoarthritis, which may resolve in a few weeks. The most frequently involved joints are knees, followed by ankles, wrist and elbows. Back pain is rare and sacroiliac joint involvement is not part of the disease. Patients with BD and arthritis also have more acne lesions. Furthermore, patients with arthritis and acne lesions have significantly more enthesopathy scores. Synovial fluid is commonly inflammatory, but has a good mucin clot. Myositis can be seen rarely.²⁹

The prevalence of neuro-BD among patients was 14.3%, which is much higher when compared to 5.3% reported in a similar prospective study. The study, which was carried out in Baghdad city for one year, included 323 patients with BD.³⁰ Central nervous system disease occurs in 5%-10% of patients in the form of either parenchymal brain involvement (80%) or non-parenchymal disease (20%).²⁹ Male predominance is more pronounced among patients with neurological involvement (about 3 or 4 males for every female), and it usually begins about 5 years after the onset of common symptoms of BD.³¹

Gastrointestinal manifestations were uncommon, with overall prevalence of 7.6%. Gastroduodenitis was seen in 2.7%, peptic ulcers in 1.5%, acute on chronic diarrhea in 2%, rectal bleeding in 0.8%, and abdominal pain mimicking a surgical acute abdomen in 1.7%. The Iranian study by Shahram et al. has reported the rare occurrence of gastrointestinal involvement of BD and vasculitis of the terminal ileum and ileocecal region.³² Gastrointestinal lesions are usually found in the ileocecal region and the symptoms are often difficult to distinguish from that of inflammatory bowel disease. The symptoms include abdominal pain, diarrhea, melena and perforation.²⁷

Prevalences of vascular involvement (arterial and venous) noted in different countries were: 8.9% in Iran, 8.9% in Japan, 7.7% in China, 1.8% in Korea, 13% in Germany, 17% in Turkey, 20% in Morocco, 25% in Tunisia (only venous thrombosis), 32% in the UK, and 10% in the US.³³ Arterial complications occur in 1%-7% of BD patients. Men are much more likely to be affected with arterial disease. Reduced brachial arterial wall flow-mediated dilatation after ischemia and increased carotid artery intima-media thickness have been found in BD patients, suggesting that microvascular dysfunction is universally present. The elevated levels of endothelin-1, a vasoconstrictor peptide,

may contribute to vessel dysfunction in BD.³⁴

Venous system involvement constitutes approximately 85% of the vascular involvement. Involvement of the venous system is most frequently seen in the form of thrombophlebitis. Thrombophlebitis can affect veins of the lower extremity, superior vena cava, and inferior vena cava. The risk of pulmonary emboli is very low in BD patients, as the deep vein thrombosis occurs mainly in inflamed veins. The most serious venous complications in BD are superior vena cava syndrome and Budd-Chiari syndrome. Venous occlusion is most frequently seen at the level of superior vena cava.³⁵

In the current study, the serum MDA level was found to be significantly increased in patients with BD than healthy controls. This result may indicate an active role of ROS in the pathogenesis of BD. Mahgoub *et al.* (2010) have indicated that lipid peroxidation has invariably been found to be accelerated in both plasma and erythrocytes of patients with BD, and is characterized by increased MDA levels or *in vitro* low-density lipoprotein oxidation.³⁶ Several studies suggest that elevated ROS production in patients with BD is associated with increased cell and tissue injury and attack double bonds in polyunsaturated fatty acids. This in turn results in lipid peroxidation and further oxidative damage.⁹

Meanwhile, the results of this study showed that the serum NO level was significantly reduced in patients with BD than healthy controls. Together with increasing MDA levels, it further describes the mechanism of BD development and the occurrence of excessive NO production during various rheumatic diseases.³⁷ On the other hand, there have been conflicting reports about serum NO concentrations in patients with BD. Some authors have reported significantly higher NO levels in patients with active disease than in patients with inactive disease or control subjects. Contradictory findings have been reported by other researchers and reported that decreased NO levels in active BD could be attributed to the rapid transformation of NO to peroxynitrites.

Genetic factors may play a role in the reduction of NO. Notably, BD-associated polymorphism of eNOS might be responsible for the reduced NO activity, thereby contributing to the development of endothelial abnormalities and thrombotic complications in these patients.³⁸ Furthermore, reduced levels of NO due to eNOS dysfunction I have been found in BD patients, particularly in those with active

disease.¹⁶ Low levels of NO could explain the vasculitic nature and thrombotic tendency of BD. Nakao *et al.* have reported that eNOS gene polymorphism is linked to BD susceptibility in Korean, Italian, and Turkish populations.³⁹

Previous studies have concluded that the reduction of NO in patients with active BD has a critical role in the development of the endothelial abnormalities and thrombotic tendency.^{14, 15, 40} The reduced levels could be attributed to increased intravascular oxidative stress and consumption of NO. Additionally, it is plausible that increased free radical production through oxidative stress, which occurs in BD, might be involved in events leading to the suppression of eNOS activity in endothelial cells.

The present study showed a significant increase in total number of granulocyte due to an elevation in the number of neutrophils, which is commonly found in BD patients with conjunctival ulcerations and intestinal lesions.⁴¹ While, a significant decrease in lymphocyte count was noted in patients with BD in compared to healthy controls. On the other hand, the total number of WBCs and monocyte count were insignificant between the groups. There are a few studies linking total WBCs count with lymphocyte count in BD. The reduction of lymphocyte count may be due to its infiltration to the site of tissue lesions, causing a subsequent decrease in the number of lymphocytes in the peripheral blood. A similar mechanism was noted by Onder and Gurer (2001).⁴² The researchers clarified that lymphocytes play a significant role in generating neutrophil hyperfunctions and chemotaxis in BD.

Most of the studies consider neutrophil as a major contributing factor in the pathogenesis of BD. Lesions of BD are typically seen in small blood vessels and these histopathological findings are marked by exudative inflammation and leukocyte infiltration to the surrounding tissues. Therefore, extensive research efforts have been focused on exploring the inflammatory reactions and the role of neutrophil functions in the etiopathogenesis of BD. Pathologically enhanced neutrophil functions including chemotaxis, phagocytosis, active oxygen radical production and lysosomal secretions have been demonstrated in BD patients. The excessive generation of the active oxygen species by activated neutrophils has been suggested to cause tissue injury.⁴³ It has been pointed out that oxidative stress abnormalities mediated by neutrophil may play a major role in BD pathogenesis.⁴⁴ Furthermore, classification of BD as a neutrophilic vasculitis has been

proposed and the concept of the neutrophilic phlebitis has been advocated.

Considering the elevated levels of ESR in BD patients, we suggest that this factor might be related to the pathogenesis of the disease. Blood ESR level was significantly higher in patients with active ocular, inactive ocular, and nonocular BD compared to the control group. The active ocular and nonocular BD groups were found to have significantly elevated ESR levels compared to the inactive ocular BD group.⁷ When neutrophils and macrophages are stimulated by pathogens, cytokines and other inflammatory mediators such as C-reactive protein (CRP), are released from granules into the cytoplasm and they play a major role in destroying phagocytosed materials. CRP and ESR levels are sensitive markers of inflammation and reflect the activity of BD.¹¹

In conclusion, the present study findings indicate that MDA and NO have roles in the pathogenesis and progression of BD and their implication in management of BD has to be explore further.

Competing interests

The authors declare that they have no competing interests.

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Correspondence: Trifa A. Abdulghafur Rahimi, Zanko New Village, 218-D, Erbil, Kurdistan Region, Iraq
trifarahimi@gmail.com

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