

The levels of nitric oxide and metabolites in Behçet's disease

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Aim: Endothelial damage and dysfunction are held responsible for the etiopathogenesis of Behçet's disease (BD). In the present study, we aimed to investigate the relationship of BD with nitric oxide (NO), asymmetrical dimethylarginine (ADMA), symmetrical dimethylarginine (SDMA) and L-arginine levels, which are known to play a role in the pathogenesis of vasculitis.

Materials and methods: Sixty patients enrolled in the study were allocated to groups as follows: active/inactive patient groups; the patient group with active/inactive vessel involvement, and mucocutaneous patient group without vessel involvement.

Results: NO, SDMA, ADMA levels of the active patients and SDMA and ADMA levels of the inactive patients were found to be significantly higher than those of the healthy controls. SMDA and ADMA levels in the group with active vessel involvement; NO, SDMA, ADMA, and L-arginine levels in the group with inactive vessel involvement; and, NO, SDMA, and ADMA levels of the mucocutaneous group were significantly higher than those of the healthy controls. However we detected no statistically significant difference among the patient groups.

Conclusion: These criteria cannot be utilized in evaluating the activity of the disease and in predicting vessel involvement.

Key words: Behçet's disease, nitric oxide, asymmetrical dimethylarginine, symmetrical dimethylarginine, L-arginine

Behçet hastalarında nitrik oksit ve metabolitlerinin düzeylerinin araştırılması

Amaç: Behçet hastalığının (BH) etiopatogenezinde endotel hasarı ve fonksiyon bozukluğu suçlanmaktadır. Bu çalışmada vaskülit patogenezinde rolü olduğu bilinen nitrik oksit (NO), asimetrik dimetilarginin (ADMA), simetrik dimetilarginin (SDMA) ve L-Arjinin düzeyleri ile BH aktivitesi arasındaki ilişkinin incelenmesi amaçlandı.

Yöntem ve gereç: Çalışmaya alınan 60 olgu aktif/inaktif hasta; damar tutulumu açısından da aktif/inaktif ve damar tutulumu olmayan mukokütanöz olarak gruplandırıldı. Nitrit ve nitrat ölçümleri spektrofotometrik; diğer parametreler HPLC cihazında florimetrik olarak ölçüldü.

Bulgular: Aktif hastaların NO, SDMA, ADMA seviyeleri ile inaktif hastaların SDMA ve ADMA seviyeleri sağlıklı kontrol grubuna göre yüksek bulunurken, incelenen parametreler yönünden aktif /inaktif hasta grupları arasında anlamlı bir fark yoktu. Damar tutulumu aktif grubun SMDA ve ADMA seviyeleri; damar tutulumu inaktif grubun NO, SDMA, ADMA, L-arjinin seviyeleri ile mukokütanöz grubun NO, SMDA, ADMA seviyeleri sağlıklı kontrol grubuna göre anlamlı derecede yüksek bulundu. Ancak, hasta grupları arasında anlamlı bir fark saptanmadı.

Sonuç: Çalışmada incelenen parametrelerin, hasta gruplarında sağlıklı kontrol grubuna göre anlamlı düzeyde yüksek olmasına rağmen, hasta grupları arasında anlamlı bir fark olmaması; hastalığın aktivitesini değerlendirmede ve damar tutulumunu göstermede yararlı birer kriter olarak kullanılamayacağı kanaatine varıldı.

Anahtar sözcükler: Behçet hastalığı, nitrik oksit, asimetrik dimetilarginin, simetrik dimetilarginin, L-arjinin

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Introduction

Behçet's disease (BD) is a multisystem inflammatory disorder, currently classified as vasculitis. Small-vessel vasculitis is the pathological basis of the multiorgan involvement that results in protean clinical features (1,2).

The exact etiopathogenesis of BD remains to be elucidated. Like most of the vasculitic pathologies, the endothelial damage and dysfunction are apparently associated with the etiopathogenesis of BD. Due to vasculitis and inflammatory response, impairment of endothelial function occurs or is accompanied by endothelial damage. In the active stage of the disease, a high systemic inflammatory activity is observed in the circulation or the vascular tissue (3-5).

Nitric oxide (NO), a molecule synthesized by endothelial cells, plays a pivotal role as a regulator during the onset of immunological and inflammatory reactions (6,7). NO is known to have prominent effects on cellular cytotoxicity and leukocyte migration (8).

Dimethylarginines, which exist as symmetric and asymmetric molecules, are analogues of L-arginine (6). Asymmetrical dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthesis. An increased plasma ADMA level has been observed in vascular diseases and is considered to be a vascular risk factor (9,10). ADMA inhibits the production of NO in cultured endothelial cells and isolated blood vessels (11). Although studies have indicated an association between high ADMA levels and endothelial dysfunction (9,12), its role in the pathogenesis of BD is unclear. The increased level of ADMA in BD patients is thought to be associated with increased synthesis and/or decreased breakdown of ADMA molecule.

It is suggested that oxidative stress can cause the level of ADMA to augment, increasing methylation of L-arginine and inhibiting the enzymes breaking ADMA down (5,6). It has been shown that the parameters indicative of oxidative stress increase in BD (13). Therefore, oxidative stress can be blamed for the increased ADMA levels in BD.

Symmetrical dimethylarginine, one of the 3 types of methylated arginine, is an isomer of ADMA (14). L-arginine is a precursor of NO synthesis. Changes in L-arginine/NO synthesis pathway could lead to endothelial dysfunction (15).

Today, practical laboratory parameters that indicate the activity of BD and particularly vessel involvement do not exist. Because of the fact that endothelial damage and dysfunction are held responsible for the pathogenesis of BD; NO, SDMA, ADMA and L-arginine levels in BD patients with vascular involvement were primarily investigated in the present study. In the current study, it was aimed to determine the relationship between BD and serum levels of NO and its metabolites, which have a critical role in immune reactions, in active and inactive periods of BD.

Materials and methods

The study was conducted in patients who were being followed up in Firat University Medical School, Dermatological and Venereal Diseases Clinic and age- and gender-matched healthy controls.

Sixty patients that were diagnosed with BD in accordance with the criteria of the International Study Group (ISG) for BD were enrolled in the study (16). The control group consisted of 24 healthy volunteers precisely matched for age and sex. For the present study, the approval of the Ethics Committee and informed consent forms from all patients and controls were obtained.

The patients were evaluated regarding the presence of symptoms and findings related to BD. At the time of the study, the patients who met at least 1 criterion of ISG for BD were considered to be in the active stage of the disease. The patients who had been free of lesions for the previous 30 days or more were taken as having inactive BD. In addition, the patients with at least one of deep vein thrombosis, superficial migratory thrombophlebitis, or large vessel involvement were considered as patients with active vessel involvement; and those with previous history were considered as patients with inactive vessel involvement. The patients without active or inactive vessel involvement constituted the mucocutaneous group.

Blood collection was performed in the morning hours (0800-1000) after overnight fasting and 30 min of supine rest. The samples were centrifuged at 3500 rpm for 10 min to obtain plasma and serum. The obtained samples were kept at -80 °C until

analysis. Nitrite and nitrate levels were measured spectrophotometrically according to a modified method of Cortas et al. (17). Levels of L-arginine, SDMA, and ADMA were measured fluorometrically by a HPLC apparatus (high performance liquid chromatography) using EUREKA kit (Head Quarter: Via E. Fermi 25 60033 Chiaravalle (AN) Italy) with programmable fluorescence detector.

Statistical analysis

SPSS v.12.0 was used to evaluate the obtained data. All values were given as mean \pm standard deviation. Mann-Whitney U test was used to compare the data obtained from patient and control groups. In addition, Kruskal-Wallis test was used for triple comparison of the data obtained from active, inactive, and control patients. Paired comparison of the groups in statistically significant results was performed using the Mann-Whitney U test. And a P value of <0.05 was regarded as statistically significant. The Chi-square test was used for evaluating the difference of gender between patients and controls.

Results

Of the 60 patients enrolled in the study, 32 were male and 28 female. The mean age of the patients was 37.28 ± 9.4 years ($n = 60$) and that of the control group was 35.70 ± 9.9 years ($n = 24$). Of the patients, 42 (70%) were active and 18 (30%) inactive. No

statistically significant difference in age and gender was found between control and patient groups ($P > 0.05$). Vascular involvement was seen in 42 (67.7%) of the patients with BD, 17 (28.4%) and 25 (39.3%) of whom were active and inactive, respectively.

When the mean NO, SDMA, ADMA values of the group with active patients were compared with those of the control group, the results of the patient group were found to be statistically significantly higher than those of the control group. A statistically significant difference was detected in SDMA and ADMA parameters between inactive patients and the controls (Table 1).

SDMA and ADMA levels of the patient group with active vessel involvement; NO, SDMA, ADMA, and L-arginine levels of the patient group with inactive vessel involvement; and, NO, SDMA, ADMA levels of the group with mucocutaneous patients were observed to be statistically significantly higher than those of the healthy controls. No statistically significant differences were detected in these parameters among the patient groups ($P > 0.05$) (Table 2).

BD is a multisystemic disease. Therefore, when the patients were evaluated in terms of organ involvement, while the mean level of serum ADMA in patients with ocular involvement was significantly higher compared to non-ocular involvement ($P = 0.02$), there were no significant difference in the other

Table 1. NO, SDMA, ADMA, and L-arginine values of the control and patient groups.

	Patient groups			P		
	Active patients (n = 42)	Inactive patients (n = 18)	Control (n = 24)	Active / Inactive	Active / Control	Inactive / Control
NO ($\mu\text{mol/L}$)	570.77 ± 12.76	528.88 ± 22.10	482.86 ± 14.75	NS	0.001	NS
SDMA ($\mu\text{mol/L}$)	7.81 ± 0.50	6.71 ± 0.52	4.00 ± 0.15	NS	0.001	0.001
ADMA ($\mu\text{mol/L}$)	55.02 ± 2.62	48.36 ± 2.79	5.02 ± 0.16	NS	0.001	0.001
L-arginine ($\mu\text{mol/L}$)	109.86 ± 13.90	86.69 ± 17.93	71.16 ± 1.71	NS	0.040	NS

NS: Not significant

Table 2. Plasma NO, SDMA, ADMA, and L-arginine values of patients regarding vascular involvement.

Plasma constituents	Patient groups			Healthy controls
	Vessel involvement active	Vessel involvement inactive	Mucocutaneous	
NO (µmol/L)	535.32 ± 32.42	536.28 ± 17.75 ^a	576.49 ± 14.52 ^a	482.86 ± 14.7
SDMA (µmol/L)	7.58 ± 0.60 ^a	7.12 ± 0.66 ^a	7.60 ± 0.61 ^a	4.00 ± 0.15
ADMA (µmol/L)	44.60 ± 4.71 ^a	56.73 ± 4.39 ^a	54.40 ± 2.50 ^a	5.02 ± 0.16
L-arginine (µmol/L)	81.77 ± 18.61	111.42 ± 24.31 ^a	106.73 ± 15.73	71.16 ± 1.71

^aP < 0.001 as compared with control (Mann-Whitney U test)

parameters. In the active patients in terms of oral ulcers, the serum level of NO was significantly higher compared to the inactive patients (P = 0.03), but there were no significant differences in SDMA, ADMA, and L-arginine levels between active and inactive patients. There were no significant differences in serum levels

of SDMA, ADMA, NO, and L-arginine between the patients with genital ulcers, erythema nodosum-like lesions, CNS involvement, gastrointestinal involvement, positivity in the pathergy test, and joint involvement and the patients without these findings (Table 3).

Table 3. The relationship between organ involvements, systemic involvements, and serum levels of NO, SDMA, ADMA, and L-arginine.

		NO (µmol/L)	SDMA (µmol/L)	ADMA (µmol/L)	L-arginine (µmol/L)	P
Ophthalmic involvement	Positive	563.4 ± 84.5	7.64 ± 2.03	63.97 ± 18.89*	113.2 ± 92.0	*P = 0.02
	Negative	557.2 ± 88.8	7.45 ± 3.19	51.09 ± 14.59*	101.0 ± 85.9	
Oral ulcer	Positive	583.2 ± 65.9**	7.92 ± 3.81	55.00 ± 15.49	117.3 ± 96.4	**P = 0.03
	Negative	536.2 ± 98.6**	7.09 ± 2.13	51.29 ± 16.14	90.2 ± 75.3	
Genital ulcer	Positive	590.7 ± 68.0	7.29 ± 2.61	46.77 ± 11.23	72.1 ± 34.3	P > 0.05
	Negative	550.9 ± 90.3	7.52 ± 3.15	54.43 ± 16.44	109.8 ± 92.8	
Erythema nodosum-like lesions	Positive	537.7 ± 102.3	6.86 ± 2.13	48.15 ± 12.06	71.5 ± 20.9	P > 0.05
	Negative	560.0 ± 86.9	7.53 ± 3.11	53.47 ± 16.13	105.7 ± 89.3	
Acneiform lesions	Positive	581.7 ± 99.7	8.53 ± 3.09	53.93 ± 19.49	110.1 ± 99.9	P > 0.05
	Negative	548.8 ± 81.6	7.06 ± 2.95	52.66 ± 14.36	100.0 ± 81.2	
Pathergy test	Positive	497.3 ± 82.1	7.57 ± 4.28	49.62 ± 15.36	66.4 ± 0.6	P > 0.05
	Negative	560.3 ± 87.6	7.48 ± 3.04	53.14 ± 15.95	104.1 ± 87.4	
Joint involvement	Positive	568.8 ± 70.5	6.37 ± 2.04	52.03 ± 8.30	96.4 ± 71.1	P > 0.05
	Negative	557.2 ± 89.4	7.58 ± 3.10	53.11 ± 16.38	103.5 ± 87.9	
CNS involvement	Positive	632.7 ± 128.1	6.85 ± 1.37	45.00 ± 18.15	133.7 ± 122.8	P > 0.05
	Negative	555.6 ± 86.2	7.50 ± 3.08	53.30 ± 15.84	101.8 ± 85.9	
GIS involvement	Positive	582.9 ± 116.4	6.61 ± 0.65	33.60 ± 7.12	52.1 ± 1.6	P > 0.05
	Negative	557.3 ± 87.5	7.51 ± 3.08	53.69 ± 15.63	104.6 ± 87.1	

Discussion

Nitric oxide contributes to antithrombotic features of the vascular wall through its functions, such as thrombocyte adhesion and aggregation, the adhesion of monocytes and leucocytes to endothelium, vascular smooth muscle proliferation, and the accumulation of superoxide radicals on the vessel wall (6,18).

Various studies have demonstrated that both increased and decreased NO levels may lead to increased damage (10,11,13). Increased productions of NO have been reported in various inflammatory diseases, such as rheumatoid arthritis, SLE, Sjörgen syndrome, vasculitis, and osteoarthritis (19). In 3 studies from Turkey, conducted in patients with BD, serum NO levels were found to be statistically significantly higher than healthy controls (20-22) whereas in a study in 21 BD patients, Örem et al (23) reported NO levels to be statistically significantly lower than those of healthy controls.

In the studies conducted by Evereklioglu et al. (24) and Yildirim et al. (25), to investigate the relationship between plasma NO levels and disease activity in patients with BD, plasma NO levels in the active patient group was found to be significantly higher than those of the inactive patient group. They have suggested that the increased plasma NO levels in the active stage of the disease might be responsible for the inflammatory process in BD and NO could be used as an activity criterion during the follow-up of the disease, indicating the severity. However, Durukan et al. (26) did not detect any statistically significant relationship between the activity of uveitis and serum NO levels in BD patients with uveitis and concluded that NO could not be used as an activity criterion in the follow-up of the disease.

In the present study, plasma NO levels were significantly higher in the active BD patients than the healthy controls, but there was no significant difference between active and inactive; and, inactive and control groups. In terms of vascular involvement, a significant increase was observed in the mucocutaneous and inactive patients compared to the control group

Our findings indicate that NO is increased in BD, which however cannot be a criterion for activity and vascular involvement. Hence, our results show consistency with those of Durukan et al. (26).

Şahin et al. (6) found that plasma ADMA level in BD patients with vascular involvement was significantly higher than that of the group of patients with mucocutaneous involvement and that of the healthy controls. In the present study, plasma ADMA levels were found to be significantly higher in all patient groups (active, inactive, and mucocutaneous) than that of the control group, whereas no significant difference was observed when the patient groups were compared among themselves.

Given that BD is vasculitis, ADMA levels are increased during inflammatory process and our results are consistent with those of other studies.

Symmetrical dimethylarginine, one of the 3 types of methylated arginine, is an isomer of ADMA. Because of the fact that SDMA has no effect on the activity of nitric oxide synthase, ADMA is regarded as a major type of endogenously formed methylarginines, which inhibit the activity of nitric oxide synthase (6). Therefore, ADMA levels were reported to be increased with SDMA levels in diseases with vessel involvement (27,28).

In the present study, plasma SDMA levels in all patient groups were found to be significantly higher than those of the control group, whereas there was no statistically significant difference among the patient groups. Depending on the fact that SDMA has no effect on the activity of nitric oxide synthase (27), it verifies the view that this parameter exerts its effect through different mechanisms from those that ADMA does and they are probably independent of NO in the pathophysiology of BD.

To the best of our knowledge, there is no report on plasma levels of L-arginine, which is a precursor of NO synthesis in BD. Kielstein et al. (29) reported that L-arginine levels in end-stage renal failure patients with atherosclerosis were significantly higher than those of patients with no atherosclerosis. In the present study, L-arginine plasma levels reach statistical significance between active BD patients and control patients, and in terms of vessel involvement between inactive patients and control groups. This situation is not fully associated with the pathology of vessel, and makes us consider that it is associated with the increased inflammation in the active stage of the disease.

BD is a chronic inflammatory systemic vasculitis, involving multiple systems. There are contradictory reports on ADMA level in some diseases with inflammation. Yoshida et al. (30) investigated the levels of ADMA and pigment epithelium derived factor (PEDF) in aqueous humour in the eyes of patients with infectious and non-infectious acute uveitis and patients with cataract. In that study, they determined that the levels of ADMA and PEDF were significantly higher in patients with uveitis compared to control group and that there was a positive correlation between the levels of ADMA and PEDF in both groups. They suggested that it was associated with inflammation.

Similarly, Sugai et al. (31) investigated the levels of ADMA in serum and aqueous humour of the eyes of diabetic patients and its relationship with the severity of retinopathy. In that study, they reported that the levels of ADMA in both serum and aqueous humour were significantly higher in the diabetic patients with particularly severe retinopathy compared to non-diabetic patients and that the levels of ADMA of serum and aqueous humour were not correlated in both groups.

In contrast to the studies given above, Blomster et al. (32) reported that there were no significant increases in the levels of ADMA, SDMA, and arginine compared to control in patients with exfoliation syndrome, whereas that there was a significant increase in the SDMA level and a significant decrease in arginine, which are correlated with serum homocysteine level, in patients with exfoliative glaucoma. They concluded that SDMA is an important marker for the development of hyperhomocysteinemia in patients with exfoliative glaucoma.

In the current study, we determined a significant increase in the serum ADMA level in patients with eye involvement. We think that both the inflammatory feature of the disease and the uveitis may a play role in that increase.

In our study, the mean serum NO level was found significantly higher in patients with active oral ulcers compared to inactive patients. There might be several reasons for the significant increase in serum NO level in patients with active oral ulcers. NO is formed from L-arginine by nitric oxide synthase enzyme. This

enzyme has 3 different isoforms, namely neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS-NOS-3). iNOS enzyme produces NO, being induced by exposure to cellular cytokines including macrophage, endothelial cells and smooth muscle cell and bacterial products, such as endotoxins and lipopolisaccarides. Activity of iNOS is long lasting and causes the production of a lot of NO, since its activation is not Ca^{2+} and calmodulin dependent. If the enzyme is induced, production of NO lasts for hours, even days (33,34).

The bacterial flora of the mouth may change nitrate in the saliva into nitrite and then NO. On the other hand, ingested nitrite in the saliva can turn into NO in the acidic medium of the stomach. Bacterial infections, particularly streptococcal infections, are blamed for the oral ulcers observed in BD. As a result, these bacteria, on the one hand, cause ulcerations, and on the other hand, induce NO synthesis for a long time (35). In this case, it is not surprising for NO level to be higher in BD patients with oral ulcers than those without oral ulcers.

Yıldırım et al. (25) reported that NO level in active BD patients was significantly higher than those in inactive BD patients and patients with *recurrent aphthous stomatitis* and healthy controls and that there was no significant difference between inactive patients and patients with recurrent aphthous stomatitis. In the current study, we found that NO levels were significantly higher in patients with oral aphthous compared to those without oral aphthous. In addition, the presence of oral aphthous in 46.7% of the patients appears to suggest that it may be a reflection of the increase of NO in patients with BD.

In conclusion, the parameters in question were significantly higher in the patient groups compared to the healthy control group. However, it was concluded that these parameters cannot be used as effective criteria in evaluating the disease activity and predicting the vessel involvement because there was no statistically significant difference among the patient groups.

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