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Asymmetric dimethylarginine levels in Behçet's disease

[Behçet hastalarında asimetrik dimetilarginin düzeyleri]

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ABSTRACT

Objective: Behçet's disease (BD) is a chronic, inflammatory, multisystem vasculitis and the etiology is not yet fully understood. Nitric Oxide (NO) is an important molecule for the vascular system which synthesised by the Nitric Oxide Synthase (NOS) enzyme. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS. Therefore, we aimed to investigate levels of the ADMA and its relation with exacerbations in BD patients.

Methods: The subjects enrolled in this study were recruited from 34 patients with BD and 34 healthy controls. Serum levels of ADMA, arginine and citrulline were measured by HPLC with fluorescence detection. The ESR and CRP levels analyzed by routinely used methods.

Results: In contrast to information in the literature, ADMA levels were found to be decreased in BD patients when compared to control. Inversely, Arginine and Citrulline levels were significantly increased in BD patients. Increased Arginine/ADMA ratios were found in the patient group. There was an inverse relation between ADMA and CRP levels. Arginine values were also correlated CRP and Citrulline levels.

Conclusion: One of the reasons of high number of BD attacks in young age may be low levels of ADMA. Our study suggests that the reduced levels of ADMA in patients group may impose a possible preventive role to ADMA through decrease of NO-mediated inflammation and exacerbations of BD with aging.

Key Words: Asymmetric dimethylarginine (ADMA), Behçet's disease (BD), Nitric oxide (NO), Vasculitis

Conflict of Interest: Authors have no conflict of interest.

ÖZET

Amaç: Behçet Hastalığı (BH), etiyolojisi tam olarak anlaşılmamış, kronik, inflamatuvar multisistemik bir vaskülittir. Nitrik Oksit (NO), endotel hücrelerinde Nitrik Oksit Sentaz (NOS) enzimi tarafından sentezlenen, vasküler sistem için önemi olan bir moleküldür. Asimetrik dimetilarjinin (ADMA) NOS'ın endojen inhibitörüdür. Bu çalışmada BH'larında ADMA düzeyleri ve ataklarla ilgisinin araştırılması amaçlandı.

Metod: 34 Behçet hastası ve 34 sağlıklı kontrol çalışmaya dâhil edildi. Serum ADMA, arjinin ve sitrülin düzeyleri HPLC yöntemi ile saptandı. ESR ve CRP düzeyleri rutin yöntemlerle analiz edildi.

Bulgular: Literatür bilgilerine ters olarak Behçet hastalarında ADMA düzeyleri kontrol grubuna göre düşük bulundu. Bunun aksine Arjinin ve Sitrülin düzeyleri Behçet hastalarında anlamlı derecede yüksek bulundu. Hasta grubunda Arjinin/ADMA oranları yüksek bulundu. ADMA ve CRP düzeyleri arasında ters bir korelasyon vardı. Arjinin değerleri de sitrülin ve CRP düzeyleri ile koreleydi.

Sonuç: BH da genç yaşta atakların sayısının fazla olmasının sebeblerinden birisi düşük ADMA düzeyleri olabilir. Bizim çalışmamız hasta grubundaki düşük ADMA düzeylerine, yaşlanma ile BH alevlenmelerini ve NO aracılıklı inflamasyonu azaltması nedeniyle muhtemel önleyici bir rol yüklemektedir.

Anahtar Kelimeler: Asimetrik dimetilarjinin (ADMA), Behçet Hastalığı (BH), Nitrik oksit (NO), Vaskülit

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Behçet's disease (BD), described in 1937 by Hulusi Behçet, characterized by recurrent oral and genital ulcers, uveitis, mucocutaneous, articular, neurological, vascular, urogenital, intestinal and pulmonary manifestations, is a chronic, inflammatory, multisystem vasculitis [1-3]. BD is more common in the Mediterranean region along the Silk Road to Japan and its' etiology is not yet fully understood. The prevalence of BD is higher in Turkey, the Middle East and Japan, while it is less common in north European countries and America [3,4]. Endothelial damage and dysfunction, secondary to chronic inflammation can be counted as the possible etiologic factors by leading to vascular inflammation in BD [5,6]. Recurrent inflammation is one of the most features of the BD especially in younger patients. The frequency of the inflammation and exacerbation of the BD attacks decrease with age [3].

Nitric Oxide (NO), synthesized in endothelial cells, is a key molecule for the vascular system. NO occurs from amino acid L-arginine, in conjunction with citrulline by the Nitric Oxide Synthase (NOS) enzyme [7,8]. NO is one of the important mediators of the inflammation and immunity, and its functions include endothelial vasodilatation and inhibition of platelet adhesion [9]. The regulation of endogenous NO synthesis mostly regulated by asymmetric dimethylarginine (ADMA). ADMA is an endogenous potent inhibitor of NOS [10]. ADMA generation occurs after the methylation of arginine residues in proteins. Proteolysis of methylated proteins to free amino acids generates free ADMA [11]. Increased plasma ADMA levels in vascular diseases are accepted as vascular risk factor [12]. ADMA, broken down by the enzyme dimethylarginine dimethylamino hydrolase (DDAH) to citrulline and dimethylamine [13,14]. ADMA is known to be involved in the pathogenesis of vasculitis and involved in synthesis of NO [5].

Currently, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are the most widely used laboratory parameters as acute phase response in clinical practice for exacerbation of BD [15].

Considering the role of NO in vascular complications of BD, and the effects of ADMA on NO synthesis, we aimed to investigate the ADMA levels and its relation with exacerbations in BD patients.

Materials and Methods

For this study, subjects were consecutively registered from patients diagnosed with BD, according to the International Study Group criteria [16] in our rheumatology out-patient clinic. The subjects enrolled in the present study were recruited from 34 BD patients (mean age 37,54±14,3 years, 13 men), and 34 healthy controls (mean age 33,29±5,76 years, 19 men). Criteria for exclusion were as follows: (a) hypertension; (b) cardiovascular diseases, such as coronary or severe valvular heart disease and myocardial infarction; (c) diabetes mellitus, renal and hepatic diseases, active infectious diseases; (d) other known chronic inflammatory diseases. The body mass index (BMI) and blood pressure were recorded. It was also noted that whether the subjects smoke or not. Each subject was evaluated in active and inactive disease periods. At the time of examination, the presence of two or more of the following Behçet's clinical features was considered as active disease: Oral ulceration, genital ulceration, skin lesions, ocular lesions, active major vessel disease, and active major organ involvement including active gastrointestinal or neurological lesions. Subjects with one lesion or lesion-free period were regarded in inactive disease period.

The protocol was approved by the Ethics Committee of the Selcuklu Medical Faculty (24.06.2010-2010/05) and all subjects volunteered for the trial and written consent was obtained according to the Declaration of Helsinki.

Blood samples were centrifuged with 2000 x g for 10 min at +4°C, followed by serum removal for assays. All serum samples were stored at -80°C until analysis.

Measurements of ADMA, arginine and citrulline levels were accomplished by high performance liquid chromatography (HPLC), using the method described by Taner *et al* [17]. In brief, 20 mg 5-sulfosalisilic acid was added to 1 ml serum, and the mixture was left in an ice bath for 10 min. The precipitated protein was removed by centrifugation at 2,000×g for 10 min. Ten µl of the supernatant was filtered using a 0.45-µm pore size filter, which was then mixed with 100 µl derivatization reagent [prepared by dissolving 10 mg o-phtaldialdehyde in 0.5 ml methanol, 2 ml 0.4 m borate buffer (pH 10.0), and 30 µl 2-mercaptoethanol were added] and injected into the chromatographic system. Separation of ADMA, arginine and citrulline was achieved with a 150×4.6 mm interior diameter Thermo ODS column with a particle size of 5 µm (Thermo, PA, USA) using 50 mm sodium acetate (pH 6.8), methanol, and tetrahydrofurane as the mobile phase (A, 82:17:1; B, 22:77:1) at a flow rate of 1.0 ml/min. Serum levels of ADMA, arginine and citrulline were measured by HPLC (HP Agilent 1200, Agilent Technologies, Palo Alto, CA, USA) with fluorescence detection. The areas of peaks detected by fluorescent detector (excitation, 338 nm; emission, 425 nm) for quantification. 26 minutes analysis time used for the measurements. Citrulline, arginine and ADMA standard and samples peaks were eluted at 11.49, 12.17, 12.64 minutes respectively in the same run. Linearity was assessed in the range 0.1–20 µM of ADMA. The mean correlation coefficient was >0.98. The ADMA limit of quantitation (LOQ) was 0.1 µM. Analytical recovery was 96.5%, and the interassay coefficient of variation was less than 5%.

At the same time the obtained samples were used to find any correlations between ADMA, arginine, citrulline and the inflammatory markers. The ESR and CRP levels ana-

Table 1. Demographic data for BD patients and control group

	Control (n=34) Mean ± SD	Behçet disease (n=34) Mean ± SD	p
Age	33.29±5.76	37.54±14.30	Ns
Gender (Male/Female)	19/15	13/21	Ns
BMI (kg/m^2)	23.66±4.07	26.56±4.94	Ns
Smoking (+/-)	5/29	4/30	Ns
Mean blood pressure	118.9±25.83	122.3±21.4	Ns
Systolic/ Diastolic (mmHg)	/ 77±12.8	/ 82±16.5	

BMI: Body mass index.

Table 2. ADMA, Arjinin, Arjinin/ADMA ratio, Citrulline, CRP and ESR Levels of BD patients and control group

	Control (n=34) Median (min-max)	Behçet's disease (n=34) Median (min-max)	p
ADMA (µmol/l)	3.22 (1.93 - 4.79)	2.25 (0.93 – 4.82)	0.001**
Arginine (µmol/l)	88.07 (58 - 130)	116.27 (71.14 - 258.38)	0.001**
Arginine/ADMA	27.26 (14.65-50)	56.34 (20.66 - 212.65)	0.001**
Citrulline (µmol/l)	63.99 (38.81 - 111.88)	91.72 (61.81 - 162.31)	0.003**
ESR (mm/h) Median (min-max)	7 (3 - 23)	8 (2 - 100)	0.49
CRP (mg/l) Median (min-max)	3.5 (1.8 - 7.24)	4.57 (2.9 - 31)	0.025*

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; *p<0.05; **p<0.01.

lyzed by routinely used methods.

Statistical Analysis

Data were analyzed by using statistical software and presented as mean ± standard deviation for normally distributed variables, median (min-max) for non-parametric values. The distribution of the variables was analyzed with the Kolmogorov-Smirnov test. Due to non-parametric values, Mann-Whitney U test were used and Student's t test was used for the normally distributed variables. Chi-square test was used for demographic values. Correlation analysis was carried out using Pearson's correlation test. P value of <0.05 was considered statistically significant for all the tests.

Results

Demographic data were given in Table 1. There was no difference between the 2 groups in terms of age, gender, BMI, blood pressure and smoking. In order to optimize patients and control populations, arterial blood pressures were measured for all study participants since ADMA correlates with high blood pressure.

ADMA levels were found to be decreased in BD patients when compared to control (p:0.001, Table 2). In contrast to ADMA levels, arginine and citrulline levels were significantly increased in BD patients (p:0.001 and p:0,003 respectively). Consequently increased Arginine/ADMA ratios were found in the patient group (p:0.001). ESR levels were similar between control and disease while CRP levels were significantly increased in the patient group.

When patients divided according to the activity status of the disease (Table 3), CRP and ESR levels were higher in the active disease patient group (p:0.003, p:0.035 respectively). There were no differences between ADMA, arginin, citrulline and arginine/ADMA ratio between the active-nonactive disease groups.

We checked the correlations separately in the patient group and in controls. In controls, there were no significant correlations with any parameter. Only patients' correlations data shown in Table 4. While there was no significant correlation between the Arginine/ADMA ratio and other parameters in controls, a significant correlation was observed between the ratio and CRP in the patients group (r: 0,634, p:0,001).

Discussion

The main findings of the present study show increased levels of serum arginine and citrulline, and decreased levels of ADMA in BD patients. Consequently, Arginine/ADMA ratio showed a significant elevation in BD. In ad-

Table 3. ADMA, Arginine, Arginine /ADMA ratio, Citrulline, CRP and ESR Levels of BD patients when classified by the disease activity.

	Remission (n=17) Median (min-max)	Active (n=17) Median (min-max)	p
ADMA (µmol/l)	2.19 (1.06 - 4.82)	2.25 (0.93 – 4.22)	0.58
Arginine (µmol/l)	113.02 (84.17 - 225.98)	133.40 (71.14 - 258.38)	0.32
Arginine/ADMA	52.95 (20.66 - 212.65)	57.29 (22.05 - 169.71)	0.45
Citrulline (µmol/l)	88.20 (64.61 - 138)	96.11 (61.81 - 162.31)	0.78
ESR (mm/h)	5 (2 - 20)	10 (2 - 100)	0.035*
CRP (mg/l)	2.9 (2.9 - 21)	8.26 (2.9 - 31)	0.003**

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; *p<0.05; **p<0.01.

Table 4. Correlations between Arginine/ADMA ratio, Citrulline, CRP and ESR in BD patients.

	Citrulline	ESR	CRP
Arginine/ADMA	r:-0.088	r:0.194	r:0.634*
Citrulline		r:0.046	r:-0.044
ESR			r:0.142

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; *p<0.01.

dition to that, there were a negative relationship between the levels of ADMA and CRP, which used as an indicator of disease activation. Arginine levels showed a significant correlation with CRP. ESR and CRP levels have been the most widely used laboratory assessments in the routine clinical practice as indicators of BD activation [15].

Due to extreme lability of NO and measurement of nitrite-nitrate (constant products of NO) does not always give enough precision, we have analyzed stable molecules such as ADMA and arginine. Low level of ADMA can not play a preventing role in NO-mediated inflammation due to the negative correlations between ADMA and CRP in BD.

Evereklioğlu *et al* [18] reported that high levels of NO are proportional to the activity of the disease, and they showed that NO plays a role in increased inflammation during the activity. Other studies have also shown an increase in NO levels of urine and synovial fluid in BD [19-20]. Vascular endothelial growth factor levels are shown to be increased in patients with BD [21] and it has been suggested to be caused by an increase in NO. According to our data low levels of ADMA may also contribute to NO increase in BD. In addition the inflammation markers and cytokines such as TNF, IL-2, IL-6, IL-8, IL-17 and IL-18 are increased in the attack period of BD. This increment in cytokines often associated with high levels of NO.

In a review, Erbil *et al* reported that complexity of formation of ADMA, and expressed that when cardiovascular risk increases also the increase in plasma ADMA concentration can be seen in many cases. But additionally they indicated that connection of protein degradation degree with the formation of ADMA is not known and ADMA is not the only factor in the pathogenesis of cardiovascular disease, probably the interactions of protein methylation, DDAH, ADMA and NO pathways are contributed to pathogenesis [11].

In this study decreased levels of ADMA and increase in Arginine/ADMA ratio in patients with BD have shown that arginine was not converted into ADMA or converted to other metabolites rather than ADMA such as citrulline and NO. Also increased citrulline levels with decreased ADMA may indicate an increase in catabolism of ADMA by the DDAH enzyme in BD. While inhibition of NOS was reduced because of low levels of ADMA in BD, NO can be synthesized more as a consequence. Our study imposes a preventive role to ADMA through decrease NO-mediated inflammation and exacerbations of BD.

Increase in arginine levels may be due to increase in protein catabolism in BD. Increased arginine also correlates with increased CRP levels. Possibly in an acute phase of the disease, increase in arginine levels provide more NO synthesis for vasodilatation therefore enhances inflammation. Increase in arginine levels may also contribute of elevated citrulline levels.

Increased levels of ADMA accompanied by cardiovascular risk factors such as hypertension, smoking, diabetes mellitus, dyslipidemia, insulin resistance and lead to vascular injury and increase the atherosclerosis have been reported [11,22]. BD is a systemic inflammatory vasculitis with unknown etiology. There are conflict results in literature, some studies [6,23] suggest that atherosclero-

sis may have accelerated in BD due to endothelial cell dysfunction, loss of elasticity in the arteries and increased lipid peroxidation. But in other studies, cardiovascular mortality is less common compared with the general population. A 10% cardiovascular mortality rate is given in patients with BD [24,25]. The possible reasons include; decrease in the severity of the disease over time, more frequent vascular involvement on the venous side, and mild and episodic course of inflammation in BD. One of the possible reason underlying the lower cardiovascular mortality might be high NO levels due to low ADMA levels. Frequent vascular complications associated with BD are indicated as large blood vessel hemorrhages, arterial aneurysm and Budd-Chiari syndrome [23,24]. According to our study, one of the underlying reasons of development of atherosclerosis in BD is less than other inflammatory diseases [26, 27] might be low levels of ADMA and high levels of arginine. On the other hand, Şahin *et al* [5] and Kökçam *et al* [28] found significantly higher plasma ADMA levels in 60 BD patients compared to healthy subjects. Our findings support the reports of Evereklioğlu *et al* [18] and Sahan *et al* [29] and shows that low ADMA levels may play a role under increased levels of NO. In addition to low ADMA levels, high arginine levels may also contribute high NO synthesis in patient group.

The onset of disease is in the third or fourth decade of life. The activation of BD was more frequent in younger patients, the frequency decreases with aging. Also ADMA increases with age [30], thereby younger patients have a relatively low ADMA and can produce more NO. NO may play a greater role in the process of vasodilatation and inflammation. One of the reasons of decreasing the frequency of attacks in Behçet's disease with advancing age may be ADMA.

In conclusion, our study suggests that the reduced levels of ADMA in patients group may impose a possible preventive role to ADMA through decrease of NO-mediated inflammation and exacerbations of BD. However, further studies should be performed to clearly establish the role of ADMA in the pathogenesis of the disease.

Conflict of Interest

There are no conflicts of interest among the authors.

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