Original Articles

Could there be an association between chronic brucellosis and endothelial damage?

Turhan Togan[1], Ozgur Ciftci[2], Hale Turan[1], Huseyin Narci[3], Hakan Gullu[2], Hande Arslan[1]

[1] Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Baskent University, Ankara, Turkey
[2] Department of Cardiology, Faculty of Medicine, Baskent University, Ankara, Turkey
[3] Department of Emergency Faculty of Medicine, Baskent University, Ankara, Turkey

Abstract
Introduction: In this study, we examined the effects of *Brucella* infection on endothelial dysfunction. Flow-mediated dilatation (FMD) measurement is indicator of the endothelial function, and abnormal values indicating endothelial dysfunction are accepted as the first stage of atherosclerosis.
Methodology: Twenty-four patients who had been treated for acute brucellosis two years before, and who had had no relapses in the follow-up, were prospectively included in the study, along with 30 healthy individuals in the control group. Results: While the highly sensitive C-reactive protein (hs-CRP) value was 2.42 ± 1.45 in the patient group, it was 1.72 ± 0.61 in the control group ($p = 0.025$). While the FMD value was 3.50 ± 1.58 in the patient group, it was 5.88 ± 1.88 in the control group ($p < 0.001$). While the percentage increase in FMD was 9.88 ± 4.92 in the patient group, it was 17.49 ± 6.3 in the control group ($p < 0.001$). It was observed that FMD value, the percentage increase in FMD, and basal radius were correlated with hs-CRP ($r = -0.644$, $p < 0.001$; $r = -0.558$, $p = 0.002$; $r = 0.444$, $p = 0.018$, respectively). The carotid artery intima media thickness (IMT) value was found to be 0.61 ± 0.17 in the patient group and 0.49 ± 0.12 in the control group ($p = 0.004$).
Conclusions: The abnormal FMD and IMT values observed in brucellosis patients might be an indicator of more frequent arterial dysfunction, increased cardiovascular risk, and atherosclerosis.

Key words: brucellosis; atherosclerosis; endothelial dysfunction; hs-CRP.

Introduction

Brucellosis is a zoonotic disease, frequently encountered in developing countries, that constitutes an important public health problem [1]. It can infect people especially through contaminated meat, milk, or dairy products, as well as through direct contact with the excrements and secretions of infected animals [2]. *Brucella* are small, Gram-negative and facultative, intracellular, pathogenous bacteria that invade mononuclear phagositic system cells to increase in them [3]. Brucellosis is an inflammatory disease that may involve any organ or system in the body [4]. It can lead to serious cardiovascular complications by affecting endothelial cells and provoking a strong inflammatory response. The secretion of adhesion molecules and proinflammatory chemokines during a *Brucella* infection plays an important role in the activation of the endothelial system. This inflammatory response, as well as the immunological reactions that develop against the *Brucella* antigens in the vessel wall, are responsible for the pathogenesis of the damage that results in the vascular system [5]. The dysfunction observed in the endothelial system is one of the first changes that occur in the pathogenesis of cardiovascular diseases, and is encountered in various clinical conditions [6]. The measurement technique of flow-mediated dilatation (FMD) has been developed in the last decade to assess the endothelium-dependent function of the vascular system following an occlusion in the brachial artery.

Nitric oxide (NO) is secreted and vasodilatation develops as a result of the stimulation of the endothelium, a situation that is considered to be a sign of vasomotor function. Ultrasound imaging of the reactive hyperaemia in the brachial artery is used to calculate the endothelium-dependent vasomotor function [7].

The state of the carotid arteries gives an idea about the state of the coronary arteries. In most of the patients with carotid artery obstruction, a stenosis is detected in the coronary arteries as well. For this reason, it is possible to get an idea about atherosclerosis through IMT measurement, a non-invasive and highly reproducible technique [8,9]. This means that the atherosclerotic changes in the carotid artery constitute an indicator of changes in the coronary artery as well. Carotid IMT measurement is recommended for people over the age of 45, and gives an idea about the risk of heart attack, coronary artery disease, or stroke that could possibly occur in this age group [10,11].

Abnormal endothelium-dependent vasodilatation is a diffuse process; the removal of the protective effect provided against atherosclerosis by a normal endothelium leads to abnormalities in the regulation of the vascular tonus. Moreover, this situation might be a sign of increased risk of cardiovascular disease. Endothelial dysfunction develops as the first stage of the atherosclerotic process [12].

A tight association between brucellosis and cardiovascular diseases is already known. Indeed, cardiovascular involvement is the main cause of mortality due to infection with *Brucella* spp., and most commonly manifests as endocarditis, peripheral and cerebrovascular aneurysms, or vascular complications such as arterial and venous thromboses. In this study, we investigated the effect of brucellosis in atherosclerosis progression. By using FMD and IMT results, we attempted to investigate the long-term effects of brucellosis on the endothelial functions of patients who have had this disease. In the literature, we did not encounter any previous studies dealing with the long-term links of brucellosis with endothelial dysfunction and the development of atherosclerosis.

Methodology
Study protocol
This prospective randomized clinical study was conducted in accordance with the Ethical Principles for Medical Research Involving Human Subjects as presented in the Declaration of Helsinki. It was carried out in the Application and Research Center of Baskent University, Konya, Turkey, after approval of the ethics board of the university was received. A total of 24 patients (15 males and 9 females) who had been treated for acute brucellosis two years before, and who had had no relapses in the follow-up, were prospectively included in the study, along with 30 healthy individuals (20 males and 10 females) in the control group. Patients between 18 and 50 years of age were included in the study between April 2010 and August 2012. After a full medical history was obtained for all patients, they were physically examined and their body mass indices (BMIs) were calculated. The exclusion criteria included a history of alcohol use, smoking, coronary artery disease in family anamnesis, intravenous drug addiction, pregnancy, use of antioxidants, hypertension, hyperlipidemia, diabetes mellitus, kidney or liver disease, rheumatological diseases, lung disease, and active infection.

Laboratory analysis
In the study, blood samples were received from the patients as well as from the control group to measure fasting blood sugar, alanine transaminase (ALT), creatinine, hemogram, highly sensitive C-reactive protein (hs-CRP), and the lipid profile, which comprised the total cholesterol, high-density lipoprotein (HDL), low density lipoprotein (LDL), and triglyceride levels. The blood samples were obtained after a night of fasting. They were collected in empty tubes and immediately stored on ice at 4°C. Following this, the serum samples were separated from the blood cells by being placed in the centrifuge at 3,000 rpm for 10 minutes. The diagnoses of brucellosis were made by standard tube agglutination test. A possible correlation between the percentage increase in FMD and IMT was sought on the one hand, and between BMI, systolic and diastolic blood pressure, fasting blood sugar, and leukocyte count on the other.

Brachial artery measurements
FMD measurements were made in both groups. FMD measurement is an indicator of the endothelial function in the brachial artery, and abnormal values indicating endothelial dysfunction are accepted as the first stage of atherosclerosis.

In this study, a high-resolution 7.5-MHz line-array ultrasound transducer (attached to a Hitachi EUB 6500, Osaka, Japan) was used. All the measurements were made by a specialist who was uninformed about the clinical data. The patients lay prone in a silent room with an interior temperature of 22°C. All the patients were prohibited from having drinks with caffeine and from smoking in the last two hours preceding the measurements.

For the imaging of the brachial artery, each patient's arm was immobilized in a relaxed, extended position, and the artery was scanned longitudinally 3–5 cm above the antecubital fossa.

The gain and depth adjustments were optimized to identify the lumen-vessel wall interface. After the transducer was placed in position, the skin area was marked as a reference point for later adjustments and the arm was held in the same position. Once all the measurements were completed, the inner diameter of the brachial artery was calculated, taking into account the QRS complex at the end of diastole; the average of the three consecutive measurements obtained during the consecutive cardiac cycle was also calculated and recorded. The basal measurements of the brachial artery were recorded. The sleeve was then placed on the proximal section of the brachial artery. The sleeve was inflated up to a pressure of 200 mm Hg (or up to 50 mm Hg above the diastolic blood pressure), and held at that pressure for a duration of five minutes, inducing ischemia in the forearm.

After this, the sleeve was deflated and the arterial diameter was measured 60 seconds after deflation. All these measurements were then recorded in a cassette for later analysis. The measurements were repeated while the diameter was greatest. The difference in percentage between the diameter measured after reactive hyperaemia and the basal diameter was taken as the FMD (FMD = 100 x [diameter after reactive hyperaemia - basal diameter] / basal diameter).

The data was independently analyzed by two specialists at the end of the study. The intra-observer variabilities for the measurements of brachial artery diameter and flow remained below 5%. The intra-observer intraclass correlation coefficient for the FMD value was 0.912. Endothelium-dependent dilatation was expressed in the form of a percentage change in the diameter of the brachial artery from baseline to post-reactive hyperaemia.

The measurement of carotid IMT

Carotid IMT was measured in this study by a previously described method, *i.e.*, by means of high-resolution, two-dimensional ultrasound images obtained with a 7.5 MHz linear-array transducer attached to an ultrasound machine (Hitachi EUB 6500, Osaka, Japan) [8]. While the subject lay in a supine position, longitudinal scanning was carried out from the common carotid artery to the bifurcation point. After confirming the bifurcation of the common carotid artery, the measurement of carotid IMT was performed from the far wall of the right carotid artery within 10 mm proximal to the bifurcation. On each scan, three points were measured, synchronized with the R-wave peaks on the electrocardiogram (ECG) to avoid errors that might possibly occur as a result of

variable arterial compliance. Mean carotid IMT was calculated using the six measurements made from two scans. All the measurements were performed by a researcher who was not informed about the subjects' clinical data. For offline analysis, the ultrasound images obtained were recorded on an S-VHS videotape. The intraobserver intraclass correlation coefficient for the measurement of carotid IMT was 0.937, and the interobserver coefficient was 0.912.

Statistical analysis

Statistical analysis was done using SPSS software version 10. The *t*-test was used for the independent samplings, and the Chi-square test was used for the continuous variables. In comparing the FMD values, the Mann-Whitney *U* test was used for the non-homogenously distributed data. The numerical values were expressed in the form of mean ± standard deviation (SD). Pearson's test was used to determine the correlation between FMD, IMT, and the other variables. The differences were accepted as statistically significant whenever $p < 0.05$.

Results

In this study, 54 patients were assessed; 24 of them had brucellosis and the remaining 30 were in the control group. There was no statistically significant difference ($p > 0.05$) in terms of gender or age between the patients in the brucellosis group and those in the control group (Table 1).

Similarly, there was no statistically significant difference between the two groups in terms of the total cholesterol, glucose, creatinine, ALT, LDL, HDL, BMI, basal systolic blood pressure, and ejection fraction (EF) values (Tables 1 and 2). Again, no statistically significant difference was found between the brucellosis and control group patients as far as leukocyte cell distribution (neutrophils, lymphocytes, and monocytes) was concerned.

As for the statistically significant variables, the hs-CRP value was 2.42 ± 1.45 in the patient group, while it was 1.72 ± 0.61 in the control group ($p = 0.025$). Similarly, the triglyceride value was 156.64 ± 63.89 in the patient group, while it was 101.73 ± 48.96 in the control group ($p = 0.001$). The HDL value was 40.68 ± 9.56 in the patient group, while it was 47.65 ± 10.05 in the control group ($p = 0.012$). The height value was 1.71 ± 0.08 in the patient group, while it was 1.65 ± 0.07 in the control group ($p = 0.013$). The FMD value was 3.50 ± 1.58 in the patient group, while it was 5.88 ± 1.88 in the control group ($p < 0.001$). The percentage increase in FMD was 9.88 ± 4.92 in the

patient group, while it was 17.49 ± 6.37 in the control group (p < 0.001). The IMT value turned out to be 0.61 ± 0.17 in the patient group, and 0.49 ± 0.12 in the control group (p = 0.004). All the data were found to be statistically significant (Tables 1 and 2). Furthermore, the FMD value, the percentage increase in FMD, and the basal radius were found to be correlated with hs-CRP (r = -0.644, p < 0.001; r = -0.558, p = 0.002; r = -0.444, p = 0.018, respectively). The IMT values were also highly correlated with hs-CRP (r = 0.466, p = 0.014).

Discussion

There is increasing evidence of the existence of a link between infection/inflammation and atherosclerosis [16]. Previous studies have focused on infections with *Chlamydia pneumoniae*, *Cytomegalovirus*, herpes simplex virus, *Helicobacter pylori*, and periodontitis in this context [13,14]. In contrast to these, various other studies have challenged the causal role of infectious agents in atherogenesis [15,16]. What current evidence does suggest, however, is that atherosclerosis may indeed develop in response to inflammatory stimuli. Accordingly, common or

uncommon infections could well represent a risk factor in this regard. Some of the mechanisms that might be involved in cases of atherogenesis induced by infectious agents include a local increase of proinflammatory cells, a local effusion of endotoxins, an autoimmune reaction, a systemic cytokine release, or changes in the lipid metabolism [17]. The cytokine-induced changes that take place in the lipid and lipoprotein metabolisms on account of infection and inflammation are similar [18].

An inflammatory response or an immune reaction against the *Brucella* antigens on the vessel wall have come under consideration as possible mechanisms in the pathogenesis of vessel involvement. The activation of the endothelium in response to the *Brucella* infection, as a result of the stimulation of the secretion of adhesion molecules and proinflammatory chemokines, may also play a role in the pathogenesis. Brucellosis may lead to cardiovascular complications, affecting the endothelial cells and provoking a strong inflammatory response [5].

In the development of atherogenesis, arterial endothelial dysfunction is one of the early-stage events leading to the development of structural

Table 1. Demographic characteristics of the patients

	Brucellosis (n = 24)	Control (n = 30)	p
Gender	1.32 ± 0.48	1.62 ± 0.50	0.031*
Male/female (*n/n*)	15/9	20/10	
Age (years)	39.89 ± 9.57	36.77 ± 3.57	0.116
Height (m)	1.71 ± 0.08	1.65 ± 0.07	0.013*
Weight (kg)	78.20 ± 12.41	72.62 ± 20.61	0.239
Body mass index (kg/m2)	26.96 ± 4.66	26.57 ± 7.31	0.817
Basal systolic blood pressure (mmHg)	121.07 ± 14.99	115.00 ± 14.06	0.148
Basal diastolic blood pressure (mmHg)	78.57 ± 9.70	75.00 ± 8.59	0.175

Table 2. Patients' laboratory and echochardiographic results

	Brucellosis (n = 24)	Control (n = 30)	p
Total cholesterol (mg/dL)	182.25 ± 34.65	173.81 ± 26.06	0.314
Triglyceride (mg/dL)	156.64 ± 63.89	101.73 ± 48.96	0.001*
HDL (mg/dL)	40.68 ± 9.56	47.65 ± 10.05	0.012*
LDL (mg/dL)	110.39 ± 31.07	109.54 ± 23.60	0.909
Glucose (mg/dL)	98.46 ± 13.99	94.04 ± 6.62	0.141
Creatinine (mg/dL)	0.79 ± 0.12	0.80 ± 0.13	0.639
ALT (mg/dL)	26.14 ± 17.53	19.85 ± 11.01	0.118
Hs-CRP (mg/L)	2.42 ± 1.45	1.72 ± 0.61	0.025*
FMD	3.50 ± 1.58	5.88 ± 1.88	< 0.001*
IMT	0.61 ± 0.17	0.49 ± 0.12	0.004*
FMD (%)	9.88 ± 4.92	17.49 ± 6.37	< 0.001*
Basal diameter	36.64 ± 5.70	34.19 ± 4.23	0.078
Peak diameter	40.14 ± 5.78	40.08 ± 4.60	0.963
EF (%)	67.03 ± 8.86	68.38 ± 4.69	0.490

ALT: alanine aminotransferase; EF: ejection fraction; FMD: flow-mediated dilatation; HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein; IMT: carotid intima media thickness; LDL: low-density lipoprotein.

atherosclerotic changes. This predisposition to vasoconstriction and/or thrombosis is important for later development of obstructive atherosclerosis [19].

Endothelium-dependent vasodilatation is realized through vasodilator agents such as NO. Abnormal NO activity is linked to atherosclerosis and vasculopathy [20]. The changes in NO levels may be determined through the measurement of the nitrite/nitrate levels in the plasma or indirectly through a non-invasive technique such as FMD measurement [21]. This measurement determines the vasodilator reaction linked to the production of endothelial NO that develops in reaction to the increased shear stress. FMD is used to detect endothelial dysfunction in cardiovascular diseases as well as in the presence of the risk factors of such diseases [7,22]. IMT is measured by FMD. It has been shown in previous studies that IMT is correlated with FMD, which is a good indicator of the endothelial function [23].

Carotid IMT can be considered a general measure of the degree of severity of atherosclerosis, and increased IMT is associated with generalized atherosclerosis [24]. The link between carotid IMT and coronary atherosclerotic status has been demonstrated in several studies [25]. Carotid IMT stands today as the most established surrogate marker of coronary atherosclerosis. It is also the only noninvasive method that is acknowledged by the American Heart Association (AHA) to be an adequate surrogate marker of coronary atherosclerosis [10]. It was also suggested in a recently published study that FMD and carotid IMT taken together have still more predictive power as a surrogate marker of coronary atherosclerosis [26]. The IMT and FMD measurements in our study also give a good idea about the risk of atherosclerosis.

The link between brachial FMD and carotid IMT

It has been demonstrated by Hashimato *et al.* that there exists a significant negative correlation between carotid IMT and brachial endothelium-dependent dilation (EDD) in patients with atherosclerosis [27]. A recently published study by Kobayashi *et al.* also indicated that there is a significant correlation between carotid IMT and brachial FMD (r = -0.343, p < 0.0001). Moreover, when FMD and carotid IMT are taken together, they have yet greater predictive power as a surrogate marker of coronary atherosclerosis [26]. Similarly, Paradossi *et al.* have found out that there is a significant association between carotid IMT and brachial FMD (r = -0.28, p = 0.002), and that it remains significant after multivariate analysis (r = -

0.24, p < 0.01) [28]. The IMT and FMD values obtained in our study corroborate the results of these studies.

The hs-CRP is an acute-phase protein that is synthesized in the liver when the organ is stimulated by various cytokines, including tumor necrosis factor-alpha and interleukin-6 [29]. Increased concentrations of hs-CRP in the blood suggests the presence of a bacterial infection and high levels of hs-CRP have been reported as an early indicator of such infections [30,31]. Moreover, there are other circumstances that cause important changes in hs-CRP concentrations. Normally, the hs-CRP levels begin to rise two to three days after the stimulus, reach a peak approximately 50 hours after it, and then begin to fall again. The exact timing depends on how fast the inflammatory process develops [32].

Considering that no such study had been conducted before on brucellosis patients, we measured the hs-CRP levels and FMD simultaneously. Our study revealed the existence of a significant link between the hs-CRP and FMD values. The fact that our study revealed a difference in the hs-CRP values between the patient and control groups, with higher values observed in the former group, is an indicator of the ongoing inflammatory process. The study also showed that the endothelium-dependent vasodilatation values, as measured by FMD, were abnormal. It was observed further that FMD, one of the noninvasive means of assessing atherosclerosis, as well as the basal percentage increase and the basal diameter, were correlated with high hs-CRP values.

There is very limited information on the serum lipid profile of brucellosis patients [33]. It has been demonstrated in previous studies that the lipids are altered in infected patients. The major part of the studies on humans focused on patients with sepsis [34].

It is well known that cholesterol levels decrease during infections, but the mechanisms underlying this phenomenon are unclear. Several studies have demonstrated that there is a correlation between the reduction in cholesterol levels as well as the alterations in apolipoprotein concentrations on the one hand, and certain cytokine and acute phase protein levels on the other. Various studies have shown the existence of such correlations in patients with sepsis [35] and in neutropenic patients with fever as well as in normal volunteers after a single intravenous endotoxin injection [36]. In addition, studies of patients with AIDS [37] and critically ill surgical patients [38] have also revealed such associations. Among these are

reductions in the serum levels of total cholesterol, HDL and LDL, as well as increases in triglycerides [39]. According to current evidence, the response of the host to infection and inflammation leads to an increase in oxidized lipids in serum, and brings about LDL oxidation *in vivo*. Oxidative modification of LDL is one of the important events leading to the development of atherosclerosis [37,40]. According to the study of Apostolou *et al.*, the total cholesterol and triglyceride levels significantly increased, the HDL levels were suppressed, and the magnitude of these changes were observed to remain high over a four-month time period [40]. In our study, the triglyceride value was found to be high in patients who had had brucellosis, while the HDL value was found to be low. In contrast to the study of Apostolou *et al.*, we found that total cholesterol was normal.

The evidence at hand suggests that *Brucella* infection might be linked with an atherogenic lipid profile. Considering the lipid profile values of the two groups compared in our study, it was observed that the differences in the triglyceride and HDL values were correlated with the abnormalities in FMD measurements, and constituted determining factors in atherosclerosis.

Study limitations

In this study, the number of patients was not sufficient. Moreover, interleukin-6, tumor necrosis factor, catecholamines, and reactive oxygen species could not be taken into consideration. To test the validity of our study, we recommend epidemiological studies focusing on the link between brucellosis and coronary artery disease.

Conclusions

Brucellosis was found to be a risk factor for coronary artery disease on account of the peripheral endothelial dysfunctions that develop in patients with this infection. The abnormal FMD and IMT values observed in brucellosis patients might be an indicator of more frequent arterial dysfunction, increased cardiovascular risk, and atherosclerosis. Moreover, we believe that brucellosis contributes to the development of coronary artery disease by altering the lipid profile (reduced HDL, increased triglycerides). This suggests that brucellosis is a preparative factor in the development of atherosclerosis. When the known and unknown risk factors of atherosclerosis are taken into account, it appears that FMD and IMT measurement might constitute a sensible method for identifying individuals at a high risk of cardiovascular disease and for taking active preventive measures.

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Corresponding author

Hale Turan, MD
Baskent University Konya Medical and Research Center
Hocacihan mahallesi. Saray caddesi No: 1
Selçuklu 42080 Konya, Turkey
Phone: +90 332 2570606 ext: 2506
Fax: +90 332 2570637
Email: turanhale@yahoo.com