

Original Article

The relationship between brucellosis and vitamin D

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Abstract

Introduction: This study was aimed to determine the relationship between vitamin D and soluble vitamin D receptor (VDR) levels and brucellosis, a common infection in Turkey, in which the cellular immune system is important in the course of the disease.

Methodology: Patients who had been followed up in the Department of Infectious Diseases and Clinical Microbiology of Cukurova University Medical Faculty, having been diagnosed with brucellosis and who had no brucellosis treatment before, were enrolled in the study along with healthy controls. The participants' vitamin D and soluble VDR values were recorded. Laboratory parameters of patients and controls, clinical findings, and disease course of brucellosis patients were also noted.

Results: The mean age of the 86 brucellosis patients, of whom 38 (44.2%) were males and 48 (55.8%) were females, was 40.9 ± 18.4 years. Complicated course of brucellosis rate was found to be 29.1%. Vitamin D and VDR levels were lower in brucellosis patients at the time of diagnosis compared to control group. For males, vitamin D and VDR levels were higher in the control group than in the patient group. In males, VDR levels were higher than in females. A significant difference was not found between clinical forms of the disease and vitamin D and VDR levels.

Conclusions: Vitamin D and VDR levels were shown to be significantly lower in brucellosis patients before treatment compared to the control group. These results suggest that vitamin D could be involved in the pathogenesis of the disease.

Key words: brucellosis; vitamin D; vitamin D receptor; immune system.

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Introduction

Zoonosis is an infection caused by bacterial, viral, and parasitic pathogens, and is transmissible from animals to humans. There are more than 175 well-defined zoonotic diseases today. One of the important zoonotic infections in our community is brucellosis. Brucellosis is a zoonotic disease with low mortality despite its quite high morbidity in Turkey. The common route of infection in Turkey is consumption of unpasteurized milk, including cheese, cream, and butter [1]. Diagnosis is usually made upon isolation of the causative agent and positive standard tube agglutination test (STAT $\geq 1/160$) in the presence of clinical signs and symptoms [2]. Brucellosis is a disease that may affect all systems, has various symptoms, and sometimes leads to severe complications such as spondylodiscitis, meningoencephalitis, and endocarditis. Diagnosis and treatment may be delayed because of these reasons. The most common complications are the ones concerning the osteoarticular system. Combined antibiotherapy

should be applied for a long period of time due to intracellular placement of the bacteria; otherwise, failure to treat and relapses may be seen.

The fact that vitamin D is essential for healthy bone development and prevention of many cancer types and autoimmune, cardiovascular, and infectious diseases has been shown in many studies performed in recent years [3-6]. Previous studies have indicated that vitamin D has very important biological effects, including cell differentiation, inhibition of proliferation, and immune modulation [7]. The immune system is affected in vitamin D deficiency. The effect of vitamin D insufficiency is seen on macrophages at cellular level; macrophages cannot function in the absence of vitamin D. Thus, chemotaxis, phagocytosis, and proinflammatory cytokine production cannot be done [8]. Vitamin D performs its immunomodulatory role via the vitamin D receptor expressed by antigen-presenting cells and T cells [9].

Brucella cannot be killed by inactivated macrophages, as it is an intracellular bacterium.

Activation of macrophages is therefore important in fighting the infection. Vitamin D is known to be a mediator that plays an important role in macrophage activation [10,11]. In this study, we investigated the relationship between vitamin D and soluble vitamin D receptor (VDR) levels and brucellosis.

Methodology

Study design

A total of 86 patients over 18 years of age who had been followed up in the ward and outpatient clinic of the Department of Infectious Diseases and Clinical Microbiology of Cukurova University Medical Faculty between May 2009 and November 2010 and diagnosed with brucellosis were enrolled in the study. None of the patients with brucellosis were excluded in the study period. Patients were enrolled in the study before treatment, at the time of diagnosis. A total of 86 healthy volunteers over 18 years of age and who did not have any complain and any symptoms and signs of infectious disease, autoimmune disorder and malignancy were included as a control group. The control group was selected from hospital staff. The entire control group was tested for inflammatory and biochemical parameters and was administered the *Brucella* agglutination test. No positive *Brucella* agglutination test was found in the control group.

Approval was obtained from the ethics committee of Cukurova University Medical Faculty, and written informed consent was obtained from the patients prior to the study.

Study variables

Demographic characteristics including age, categorical age (18–39, 40–59, > 60), gender, possible infection route, time of beginning of complaints, and laboratory findings of the cases were evaluated for diagnosis and recorded.

An automatized blood culture system (BACTEC, (Cockeysville, USA) was used for bacterial identification. Whole blood count, 25 (OH) D, calcium (Ca), phosphorous (P), PTH (parathyroid hormone), AP (alkaline phosphatase), albumin, total protein, AST (aspartate aminotransferase), ALT (alanine aminotransferase), thyroid function tests, CD4 levels, CRP (C-reactive protein), ESR (erythrocyte sedimentation rate) levels and *Brucella* tube agglutination test of patients and control group were measured. Serum samples for soluble VDR of patient

and control groups were stored at -20°C to be studied once a month.

Microbiological methods

A total of 8–10 mL of patients' blood samples was inoculated into BD BACTEC Plus Aerobic/F hem culture vials for blood cultures and incubated in BACTEC 9240 (Becton Dickinson, Cockeysville, USA) automatized blood culture system at 35°C for seven days. Gram staining preparations were prepared from the blood culture vials of which positive signal was obtained by the BACTEC device and concurrent passage into 5% sheep blood Columbia agar, Mac Conkey agar and chocolate agar was performed. At the end of the 7-day incubation period by using BACTEC device for vials with no signalling, passages were made into chocolate mediums for control and no growth was considered as negative blood culture result.

Identification of isolates was done using colony morphology, Gram staining, and an agglutination test with monospecific antiserum. Urease activities of isolates seen as pale, small, Gram-negative coccobacillus with positive oxidase and catalase tests were investigated, and a pre-diagnosis of brucellosis was made. Additionally, all of the colonies were defined as *Brucella* using Gram-negative bacteria identification cards in the VITEK 2 (bioMerieux, Marcy l'Etoile, France) system. All laboratory studies were conducted in a second-level safety cabinet.

Measurement of serum VDR levels

Blood samples taken into plain tubes were centrifuged at 1,000 rpm for 15 minutes for measurement of serum VDR levels. Afterwards, plasma was separated and stored at -20°C until the day of analysis. A micro enzyme-linked immunosorbent assay (ELISA) test kit was used for measurement of serum VDR levels.

Serum 25 (OH) D measurement

Fasting blood samples were taken into EDTA tubes from the patients for detection of serum 25 (OH) D levels in morning times. Serum 25 (OH) D levels were measured daily with chromatographic method in Shimadzu LC 20AD/T series (Kyoto, Japan) high-performance liquid chromatography (HPLC) device in Central Laboratory of Balcali Hospital, Cukurova University Medical Faculty.

Definitions

Patients diagnosed as positive for *Brucella* infection were determined using the following criteria in

presence of clinical signs and symptoms: specific antibody titer of $\geq 1/160$ in serum standard tube agglutination test (STA); fourfold or greater increase in titers in tests performed in two- to three-week intervals; or growing of *Brucella* in any culture samples.

Statistical analysis

The categorical variables between the groups were analyzed using the Chi square test or Fisher's exact test. For each continuous variable, normality was checked using the Shapiro-Wilk test. Student's t test was used for the continuous variables that were normally distributed. Since the data was not distributed normally, an appropriate non-parametric test was chosen and comparisons were applied using the Mann-Whitney U test. Results are presented as mean \pm standard deviation (SD) and median (min-max), number (n), and percentage. P value < 0.05 was considered to be

statistically significant. Dataset was analyzed using the Statistical Package for Social Sciences (SPSS) version 19.

Results

The mean age of patients was 40.8 ± 18.4 (range, 18–78) and of control groups was 37.1 ± 13.4 (range, 18–80) ($p = 0.403$). The male-to-female ratio was 38/48 for patients and 43/43 for controls.

The possible source of infection was detected in 78 (90.9%) cases. Infection via the consumption of milk and dairy products occurred more frequently than other infection routes.

The duration between the onset of complaints and admission to the clinic was found to vary between one week and 55 weeks. A total of 72 (83.7%) patients were diagnosed as having brucellosis (duration of symptom onset was shorter than 8 weeks), 11 (12.8%) patients

Table 1. Distribution of the laboratory parameters of the study groups.

	Patients	Controls	P value
	Mean \pm SD Med (min-max)	Mean \pm SD Med (min-max)	
White blood cells (/mm ³)	6.2 \pm 2.6 6.0 (1.4–15.4)	7.7 \pm 2.2 7.3 (4.0–17.0)	0.0001
CRP (mg/L)	35.1 \pm 34.3 24.0 (1.9–131.0)	4.9 \pm 4.3 3.0 (1.2–23.0)	0.0001
ESR (mm/h)	23.6 \pm 23.0 16.0 (2.0–111.0)	4.9 \pm 5.4 2.0 (2.0–37.0)	0.0001
ALP (U/L)	255.8 \pm 164.5 218.5 (50.0–921.0)	185.3 \pm 50.9 175.0 (88.0–430.0)	0.003
AST (U/L)	37.8 \pm 27.8 27.0 (11.0–153.0)	22.4 \pm 6.2 21.0 (13.0–40.0)	0.0001
ALT (U/L)	32.1 \pm 24.2 25.0 (7.0–143.0)	22.2 \pm 10.9 19.5 (5.0–67.0)	0.004
Total protein (gr/dL)	7.2 \pm 0.9 7.4 (4.4–9.8)	7.6 \pm 0.5 7.6 (5.0–8.7)	0.003
Albumin (gr/dL)	3.9 \pm 0.6 3.9 (2.5–4.9)	4.7 \pm 0.3 4.7 (3.9–5.3)	0.0001
Calcium (mg/dL)	8.9 \pm 0.6 8.9 (7.4–11.0)	9.6 \pm 0.5 9.6 (8.2–11.3)	0.0001
PTH (pg/mL)	34.5 \pm 18.8 32.0 (8.0–91.0)	42.8 \pm 18.4 39.0 (13.0–97.0)	0.001
Phosphorous (mg/dL)	3.5 \pm 0.8 3.5 (1.5–5.5)	3.6 \pm 0.7 3.5 (2.3–5.9)	0.646
Hematocrit (%)	35.4 \pm 5.3 36.0 (23.0–45.0)	41.8 \pm 4.6 42.0 (29.0–49.0)	0.0001
Hemoglobin (gr/dL)	11.3 \pm 1.8 11.5 (7.0–15.0)	13.7 \pm 1.7 14.0 (9.0–17.0)	0.0001
Platelet (/mm ³)	228.3 \pm 99.9 214.5 (33.0–609.0)	242.9 \pm 65.8 238.0 (122.0–556.0)	0.133
TSH (IU/mL)	1.7 \pm 1.0 1.4 (0.0–4.5)	1.5 \pm 0.7 1.4 (0.1–3.8)	0.458
FT4 (ng/mL)	1.2 \pm 0.3 1.2 (0.1–2.7)	0.9 \pm 0.5 1.1 (0.1–2.2)	0.0001
CD4	744.1 \pm 332.5 653.0 (156.0–1697.0)	942.0 \pm 258.6 904.0 (443.0–1833.0)	0.0001

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; PTH: parathyroid hormone; TSH: thyroid stimulating hormone; FT4: free thyroxin.

were diagnosed as having subacute brucellosis (duration of 8–52 weeks), and 3 (3.5%) patients were diagnosed as having chronic brucellosis (duration longer than 52 weeks).

An STA test was performed on all patients. STA positivity was detected most commonly at titers of 1/640 (38.4%). STA test was negative in one case (1.2%), 1/40 in two (2.3%) cases, and 1/20 in one (1.2%) case. Diagnosis was made upon growing in blood cultures of those four patients.

Brucella melitensis was isolated in blood cultures of 29 (33.7%) cases.

The distributions of the laboratory parameters of the study groups are given in Table 1. A statistically significant difference could not be found between initial phosphorous, platelet, and thyroid-stimulating hormone (TSH) values in patient and control groups ($p > 0.05$). There was a statistically significant difference between white blood cell count, hematocrit, AST, ALT, AP, PTH, calcium, CRP, ESR, total protein, albumin, and FT4 (free thyroxin) levels ($p < 0.05$).

Levels of 25 (OH) D and VDR of patient and control groups are shown in Table 2. While the mean 25 (OH) D was 23.3 ± 13.2 ng/mL in patients, it was 30.2 ± 16.1 ng/mL in the controls ($p = 0.005$). The mean VDR level was found to be statistically lower in the patient group (1.21 ± 1.04 ng/mL) than in the control group (1.77 ± 1.10 ng/mL) ($p = 0.0001$). There was no significant difference between males and females both

in patient and control groups for 25 (OH) D. There was a significant difference between males and females in the control group, but no significant difference in the patient group for VDR levels.

Levels of 25 (OH) D and VDR of patients and controls based on age groups are shown in Table 3. Both 25 (OH) D and VDR were found to be statistically higher in controls than in patients in some age groups.

While there was no significant difference between patients and controls for females, there was a difference for males both in 25 (OH) D ($p = 0.050$) and VDR ($p = 0.001$).

The relationship between clinical forms of the patients and 25 (OH) D and VDR is shown in Table 4. Due to the insufficient sample size, subacute and chronic groups were combined, and after that, groups were compared. There were no statistically significant difference between groups in terms of both 25 (OH) D and VDR ($p = 0.073$, $p = 0.179$, respectively).

Discussion

This study shows that vitamin D and soluble VDR levels were low in patients with diagnosed brucellosis in all age groups when compared with a healthy population. But these parameters do not have a correlation with the course of the disease.

Vitamin D was believed to have many significant effects, including cell differentiation, inhibition of proliferation and immunomodulation except calcium

Table 2. Distribution of the 25 (OH) D and VDR levels of the study groups by gender.

	25 (OH) D (ng/mL)			VDR (ng/mL)		
	Mean \pm SD		P **	Mean \pm SD		P **
	Patient	Control		Patient	Control	
	Median (min-max)			Median (min-max)		
Total	23.3 ± 13.2 22.0 (5.0–57.0)	30.2 ± 16.1 26.0 (6.0–77.0)	0.005	1.2 ± 1.0 0.9 (0.0–4.3)	1.8 ± 1.1 1.6 (0.2–4.8)	0.0001
Female	21.9 ± 13.1 21.5 (5.0–57.0)	27.3 ± 15.6 23.0 (8.0–65.0)	0.163	1.2 ± 1.0 0.8 (0.0–4.3)	1.3 ± 0.8 1.2 (0.2–3.2)	0.238
Male	25.1 ± 13.3 22.5 (5.0–51.0)	31.5 ± 16.3 30.5 (6.0–77.0)	0.050	1.2 ± 1.0 0.9 (0.0–3.7)	2.0 ± 1.2 1.8 (0.2–4.8)	0.001
P value*	0.284	0.234		0.892	0.005	

*Between male and female; ** Between patients and controls.

Table 3. The distribution of the 25 (OH) D and VDR levels of the study groups according to age.

	25 (OH) D (ng/ml)			VDR (ng/ml)		
	Mean \pm SD		p	Mean \pm SD		p
	Patient	Control		Patient	Control	
	Median (Min-Max)			Median (Min-Max)		
18-39	$23,7 \pm 13,9$ 22,0(5,0-57,0)	$27,0 \pm 14,0$ 25,0(8,0-57,0)	0,231	$1,2 \pm 0,9$ 1,1(0,0-3,8)	$1,7 \pm 1,1$ 1,5(0,2-4,8)	0,014
40-59	$20,4 \pm 10,3$ 19,0(6,0-51,0)	$37,9 \pm 18,3$ 35,0(6,0-77,0)	0,0001	$1,4 \pm 1,4$ 0,8(0,0-4,3)	$1,9 \pm 1,2$ 2,0(0,4-4,5)	0,089
>60	$24,9 \pm 14,3$ 25,0(5,0-51,0)	$30,8 \pm 19,0$ 26,5(13,0-65,0)	0,598	$0,9 \pm 0,9$ 0,6(0,0-3,2)	$1,5 \pm 0,7$ 1,2(1,2-2,9)	0,050

Table 4. Relationship between clinical form and 25 (OH) D and VDR levels.

		N (%)	Mean ± SD Median (min-max)	P value
25 (OH) D	Acute	72 (83.7)	24.5 ± 13.6 22.0 (5.0–57.0)	0.073
	Subacute + Chronic	14 (16.3)	17.0 ± 8.6 18.5 (5.0–31.0)	
VDR	Acute	72 (83.7)	1.3 ± 1.0 1.0 (0.0–4.3)	0.179
	Subacute + Chronic	14 (16.3)	1.0 ± 1.1 0.5 (0.0–3.65)	

homeostasis and bone metabolism with the discovery of vitamin D receptors in many tissues. Its effects on the immune system are one of the issues investigated most frequently. Vitamin D plays a role in the stage of transfer to acquired immunity from natural immunity [12]. Most biological effects of 1,25 (OH)₂ D₃, the active form of vitamin D, require the presence of high-affinity VDR.

Many studies have investigated the role of vitamin D in protection against upper and lower respiratory system infections caused by various etiologic agents, mainly viral and HIV infection [13-15]. It has been reported that susceptibility to infections such as tuberculosis increased in individuals with severe vitamin D deficiency.

In vitro studies have indicated that vitamin D and metabolites may have important roles in the regulation of granulomatous reactions and can increase the ability to prevent *Mycobacteria* growth through activation of alveolar macrophages [10,16-18]. Granulomatous reactions occur as characteristic tissue responses in the ongoing fight between activated macrophages and persistent microorganisms; granulomas are formed in tissues and organs. It may be expected that vitamin D shows the same effect as in tuberculosis, as brucellosis causes a granulomatous infection. In our study, when the patient group with brucellosis was compared with the control group, 25 (OH) D levels were found to be 23.28 ± 13.21 ng/mL and 30.21 ± 16.14 ng/mL, respectively, and the difference was found to be statistically significant ($p = 0.005$). These findings support our opinion.

A soluble vitamin D receptor kit was used in our study. The manufacturer produced this kit for investigation purposes, not for diagnosis. The test allows receptor measurement in serum. As mentioned before, vitamin D receptor is present in skin, breast, hypophysis, parathyroid gland, beta cells of pancreas, gonads, brain, skeletal muscles, circulating monocytes and activated T and B lymphocytes, in addition to intestines, bones, and kidneys [19]. The tissues that

include VDR are also 1,25 (OH)₂ D producing sites [20]. A proper test for quantitative measurements of VDR as a regulator of cell growth and differentiation is still not available despite the growing findings of the importance of the VDR system. Its measurement in tissue samples is used in studies. VDR ELISA test is an easily applicable and faster test. It uses small sample size, requires little protein, and benefits from detection methods that are not radioactive. The test was found to be compatible with vitamin D levels in our patients with brucellosis and supported our theory. Human studies are needed for this test and studies comparing with tissue receptor level are also needed.

Changes are known to occur in vitamin D levels during treatment applications. These changes could not be determined in our study as measurement of values on follow-up and at the end of treatment was not included.

In Dam *et al.*'s study of 1,065 females and males with mean age of 74.6 years, the researchers found mean serum 25 (OH) D concentrations higher in males than in females (107.6 ± 29.2 nmol/L vs. 100.8 ± 33.1), although 28.6% of females and 9.3% of males were receiving vitamin D supplementation [21]. A statistically significant difference was not found in our study, although 25 (OH) D and VDR levels were found to be lower in female patients than in male patients. In the healthy control group, 25 (OH) D and VDR levels were found to be significantly lower in females than in males ($p = 0.0001$, $p = 0.001$, respectively). A disappearance of the difference between vitamin D levels in males and females observed in the normal population calls attention to the relationship between vitamin D and the disease. Vitamin D level was observed to be low in all patients with brucellosis.

Although classifying the cases as acute, subacute, and chronic is not the best approach because brucellosis is a disease that may be asymptomatic, it is still used in many studies due to ease of approach. In our study, 72 (83.7%) patients were found to have acute brucellosis, 11 (12.8%) subacute, and 3 (3.5%) chronic brucellosis. The relationship between clinical forms of the disease

and 25 (OH) D and VDR levels was studied with the thought that vitamin D and VDR levels may change over time or manifestation may be affected by vitamin D level. A statistically significant difference was not detected between disease groups in terms of both 25 (OH) D and VDR. However, it should be kept in mind that scantiness of chronic cases might not reveal a statistical difference.

Changes in serum calcium and phosphate levels cause differences in VDR expression in target tissue [22-23]. PTH plays a role in the regulation of VDR expression. Hypocalcemia and reduction in intracellular calcium amount is a factor strongly stimulating PTH excretion. PTH excretion is inhibited by 1,25 (OH)₂D₃ and hypomagnesemia. The most important effect of PTH is bone resorption providing calcium and phosphorous release. This effect of PTH requires vitamin D and magnesium levels to be normal. PTH also reduces calcium excretion from kidneys and stimulates 1, 25 (OH)₂D₃ production, which reduces PTH excretion. Although not precise, it also increases absorption of Ca⁺² from kidneys. In our study, low calcium and PTH levels were found alongside low vitamin D and VDR levels. Although the relationship between low vitamin D and hypocalcaemia was revealed in the patient group, it is interesting that PTH elevation does not accompany this. However, it does not seem possible to explain this situation with known mechanism of different immune steps caused by the infection or inhibitor inflammatory conditions (cytokine, chemokine). Inflammation may be affecting normal physiologic functions. As a result, it was shown in this study that 25 (OH) D and VDR levels were significantly lower in patients with brucellosis compared to a control group. Additionally, changes in calcium and PTH metabolism are observed during the course of the disease. All of them support the role of 25 (OH) D in the pathogenesis of the disease.

Conclusions

Our study is important as it is the first study that examined the relationship between 25 (OH) D, VDR levels, and brucellosis. Vitamin D and VDR levels were shown to be significantly lower in brucellosis patients before treatment compared to a control group. These results suggest that vitamin D could be effective in the pathogenesis of the disease.

Future studies should investigate the relationship between brucellosis and vitamin D more comprehensively. More detailed studies should be done to examine the relationship between infections or inflammation and vitamin D, PTH, and calcium cycles.

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