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Colchicine Modulates Oxidative Stress in Serum and Neutrophil of Patients with Behçet Disease Through Regulation of Ca^{2+} Release and Antioxidant System

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Abstract Behçet disease (BD) is a chronic, inflammatory, and multisystemic condition with an uncertain pathogenesis. One of the major immunologic findings in BD pathogenesis is increase in activity of neutrophil. An increase in the cytosolic free Ca^{2+} $[Ca^{2+}]_i$ concentration that induces Ca^{2+} signaling is an important step that participates in the neutrophil activation and reactive oxygen species production that leads to tissue damage in body cells. We aimed to investigate the effects of colchicine on oxidative stress and Ca^{2+} release in serum and neutrophil of BD patients with active and inactive periods. Twelve Behçet patients (6 active and 6 inactive) and 6 control subject were included in the study. Disease activity was considered by clinical findings. Serum and neutrophil samples were obtained from the patients and control subjects. Neutrophils from patients with active BD were divided into three subgroups and were incubated with colchicine, verapamil + diltiazem, and colchicine + verapamil + diltiazem, respectively. Erythrocyte sedimentation rate, leucocytes counts, serum C-reactive protein, neutrophil, and serum lipid peroxidation and intracellular Ca^{2+} release levels were higher in active and inactive groups than in the control group, although their levels were lower in active group than in inactive group. However, neutrophil Ca^{2+} release levels were decreased in colchicine, verapamil + diltiazem, and colchicine + verapamil + diltiazem groups group compared to active group. Serum glutathione, vitamin A, vitamin E, and β-carotene concentrations were lower in active and inactive groups than in the control group, although serum vitamin E and β-carotene concentrations were higher in the inactive group than in the active group. Neutrophil and serum glutathione peroxidase activity within the three groups did not change. In conclusion, we observed the importance of Ca^{2+} influx into the neutrophils and oxidative stress in the pathogenesis and activation of the patients with BD. Colchicine induced protective effects on oxidative stress by modulating Ca^{2+} influx in BD patients.

Keywords Antioxidant · Behçet disease · Calcium ion · Colchicine · Inflammation · Oxidative stress

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Behçet disease (BD) was first defined in 1937 by the dermatologist Hulusi Behçet as a three-symptom complex of recurring oral and genital ulcers and uveitis with hypopyon (Yazici et al. 2010). Over time, it was understood that BD was not a three-symptom complex but that there may be systemic involvement (Evereklioğlu 2005). BD is found in patients over a wide geographical area extending from the Mediterranean to the Far East, with the greatest distribution along the historical Silk Road (Direskeneli 2001). Turkey is the country with the highest prevalence of BD (Evereklioğlu 2005; Yazici et al. 2010).

BD is a chronic inflammatory vasculitis characterized by recurring endothelial dysfunction, neutrophil hyperfunction, and excessive reactive oxygen species (ROS) production (Kökçam and Nazıroglu 2002; Kauraki et al. 2003; Ben Ahmed et al. 2004). Neutrophils are cells that play an important role in immune response. In BD, there is an increase in neutrophil chemotaxis, phagocytosis, and other neutrophil functions in the peripheral blood and skin lesions (Direskeneli 2001; Zouboulis and May 2003).

The main factor in BD pathogenesis is inflammation, and the most significant cause of inflammation has been shown to be ROS. The ROS are the products of biological reduction reactions (Nazıroğlu 2007). Uncontrolled free radicals in a living organism cause disease and cell damage by affecting many molecules such as carbohydrates, proteins, lipids, and DNA. Glutathione peroxidase (GSH-Px), superoxide dismutase, and catalase are antioxidant enzymes which eradicate free radicals, whereas reduced glutathione (GSH) is a nonenzymatic antioxidant (Nazıroğlu 2007, 2009; Kovacic and Somanathan 2008). By activating proteinases and chemotaxis causing the production of cytokines, ROS cause tissue damage and inflammation (Erkiliç et al. 2003; Yazici et al. 2004).

All cellular functions are triggered by cellular ion changes. The basic ion Ca^{2+} is the most important trigger of these functions. Ca^{2+} is an ion that controls several physiological events such as exocytosis, secretion, and apoptosis (Ayub and Hallett 2004; Nazıroğlu 2007). Intracellular free Ca^{2+} is one of the most important ions affecting neutrophil functions. Ca^{2+} ion affects chemotaxis, adhesion, and the expression and production of the ROS and arachidonic acid products in the neutrophils. Therefore, a change in the intracellular Ca^{2+} levels in the neutrophils directly affects the neutrophil response (Bréchard and Tschirhart 2008).

Because there is no specific treatment for BD, symptomatic and empirical treatment methods are applied. Colchicine, an alkaloid traditionally extracted from *Colchicum autumnale* (meadow saffron), remains a common choice in treatment acute gout arthritis. Colchicine has been commonly used for treatment of inflammatory diseases such as familial Mediterranean fever (Modriansky et al. 2002). Colchicine is a medication in widespread use because it has been shown to be effective in BD treatment too. Although the mechanism of the effect of colchicine on BD is not yet fully understood, it has been shown to have an anti-inflammatory, antioxidant, and inhibitor effect on neutrophils (Köse et al. 1995; Kökçam and Nazıroglu 2002).

In this study, our first aim was to research the importance of the Ca^{2+} flow to the neutrophil cytosol in BD activation and the effect of colchicine treatment on the neutrophil cytosol Ca^{2+} release from intracellular stores evoked by *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) as a calcium mobilizing agonist. Our second aim was to study the dual effect of colchicine in human neutrophil cells by assessing its role on lipid peroxidation (LP) and antioxidant concentrations. Thus, the mechanisms held responsible for neutrophil activation in BD pathogenesis will be able to be understood, and new treatment methods will be able to be developed.

Subjects and Methods

Chemicals

All chemicals (cumene hydroperoxide, KOH, NaOH, thiobarbituric acid, 1,1,3,3 tetraethoxy propane, 5.5-dithiobis-2 nitrobenzoik, tris-hydroxymethyl-aminomethan, 5.5-dithiobis-2 nitrobenzoik asit, cumene-hydroperoxide, glutathione, butylhydroxytoluol, fMLP, thapsigargin′ digitonin, and ethylene glycol-bis(2-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) were obtained from Sigma-Aldrich Chemical (St. Louis, MO), and all organic solvents (*n*-hexane, ethyl alcohol) were purchased from Merck Chemicals (Darmstadt, Germany). Fura-2 acetoxymethyl ester was purchased from Promega (Eugene, OR). All reagents were analytical grade. All reagents except the phosphate buffers were prepared daily and stored at +4°C. The reagents were equilibrated at room temperature for half an hour before an analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4°C for 1 month.

Patient and Control Subjects

The study was conducted at the Biophysics Research Laboratory, Suleyman Demirel University (SDU), Turkey. The patients enrolled in the study were selected from patients attending the Dermatology Department of SDU. The study was approved by the Ethics Committee, Medical Faculty, SDU. All participants provided written consent, confirming their acceptance for giving blood through vena brachialis, and were informed about the entire experimental procedure. A diagnosis of BD was given by calculating the BD current activity index according to the criteria defined by the BD International Working Group. Patients were accepted as active with a total index score of 4 or more. According to the current activity index, the study comprised 6 active and 6 inactive patients with BD and 6 healthy individuals. The active BD patient group comprised 4 men (66.6%) and 2 women (33.3%) of mean ± SD age 39.5 ± 8.16 years. The inactive BD group comprised 2 men (33.3%) and 4 women (66.6%) of mean age 43.3 ± 5.68 years. The control group comprised 4 men (66.6%) and 2 women (33.3%) of mean age 38.6 ± 7.39 years. There was no statistically significant difference between the ages of the groups in the study.

Neutrophils were isolated by taking a venous blood sample from each individual. For all those included in the study, demographic characteristics, clinical information, physical examination findings, and laboratory test results were recorded. Informed consent was obtained from all study participants.

Three study groups were formed, as follows, with six samples in each group: group 1, neutrophils from active patients with BD were incubated with (group 1a) colchicine [5 μg/ml (10^7 cells) for 1 h] (Bollimuntha et al. 2005), (group 1b) diltiazem + verapamil [10 μg/ml (10^7 cells) for 30 min] (Shima et al. 2008), or (group 1c) colchicine + diltiazem + verapamil; group 2, inactive patients with BD; and group 3 ($n = 6$), healthy control group.

Method

After an overnight fast, blood samples of the control and patients were drawn from the antecubital vein into tubes with and without anticoagulant. Blood with anticoagulant were used for analyses of erythrocyte sedimentation rate (ESR) and leucocyte count. Serum and neutrophil samples were obtained from the blood samples. Serum and half of the neutrophil samples were stored at −33°C and were used for LP and antioxidant analysis within 1 month. The remaining neutrophil samples were used for daily $[Ca^{2+}]_i$ analysis.

Isolation of Human Neutrophils

Neutrophils were isolated from peripheral whole blood of healthy volunteers and patients with BD, as described previously (Şahin et al. 2011), by centrifugation through Ficoll. Half of the cells were stored for antioxidant analyses. The remaining cells were used for measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$). Sterile solutions used for neutrophil isolation were phosphate buffered saline from Gibco Invitrogen (Istanbul, Turkey) and 6% hydroxyl ethyl starch solution in isotonic NaCl from sterile plasma, purchased from Fresenius AG (Bad Homburg, Germany); and Ficoll-Paque PLUS from GE Healthcare Bio-Sciences (Uppsala, Sweden). The loading buffer contained HEPES (20 mM), NaCl (138 mM), KCl (6 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.6 mM), and glucose (5.5 mM), pH 7.4, and was supplemented with 2% (v/v) of autologous serum. The measuring buffers did not contain serum but were otherwise identical in composition to the loading buffer when a normal extracellular Ca^{2+} concentration was explored.

Measurement of Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$) in Neutrophils

Neutrophils were loaded with 4 μM fura-2/AM in loading buffer with 5×10^6 cells per ml for 45 min at 37°C in the dark, washed twice, incubated for an additional 30 min at 37°C to complete probe de-esterification, and resuspended in loading buffer at a density of 3×10^6 cells per ml according to a published procedure (Uğuz et al. 2009). The four groups were exposed to fMLP to stimulate ($[Ca^{2+}]_i$) release. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension at 37°C using a spectrofluorometer (Carry Eclipsys, Varian Inc, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz et al. (1985). In the experiments where a calcium-free medium is indicated, Ca^{2+} was omitted and 2 mM ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added.

Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 150 s after addition of fMLP (Espino et al. 2009; Uğuz et al. 2009; Şahin et al. 2011). Ca^{2+} release is expressed as nanomoles, taking a sample every second as previously described (Heemskerk et al. 1997).

LP Determinations

LP levels in the serum and neutrophil samples were measured with the thiobarbituric acid reaction by the method of Placer et al. (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of malondialdehyde equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of LP in the neutrophil and serum samples were expressed as μmol/g protein and nmol/ml, respectively.

GSH, GSH-Px, and Protein Assay

The GSH content of the serum and neutrophil samples was measured at 412 nm using the method of Sedlak and Lindsay (1968). GSH-Px activities of serum and neutrophil samples were measured spectrophotometrically at 37°C and 412 nm according to the method of Lawrence and Burk (1976). The protein content in neutrophil samples was measured by method of Lowry et al. (1951) with bovine serum albumin as the standard.

Serum Vitamins A, E, and β-Carotene Analyses

Vitamins A (retinol) and E (α-tocopherol) were determined in the serum samples by a modification of the method described by Desai (1984) and Suzuki and Katoh (1990). Serum samples (250 μl) were saponified by the addition of 0.3 ml KOH (60% and w/v in water) and 2 ml of 1% (w/v in ethanol) ascorbic acid, followed by heating at 70°C for 30 min. After cooling the samples on ice, 2 ml of water and 1 ml of *n*-hexane were added and mixed with the samples, then rested for 10 min to allow phase separation. An aliquot of 0.5 ml of *n*-hexane extract was taken, and vitamin A concentrations were measured at 325 nm. Then reactants were added and the absorbance value of hexane

was measured in a spectrophotometer (Shimadzu, UV-1800, Kyoto, Japan) at 535 nm. Calibration was performed using standard solutions of all-trans retinol and α-tocopherol in hexane.

The concentrations of β-carotene in the serum samples were determined according to the method of Suzuki and Katoh (1990). Two milliliters of hexane were mixed with 250 µl serum samples. The concentration of β-carotene in hexane was measured at 453 nm in a spectrophotometer.

Biochemical Analysis

Leucocyte counts were measured in a cell counter (Beckman Coulter LH 70 hematology analyzer; Beckman Coulter, La Brea, CA). ESR was measured by routine Western green methods. C-reactive protein (CRP) is one of the acute-phase proteins that increases during systemic inflammation, and serum CRP values in patients with BD were measured in a Nephelometry device (Delta Seac Radim, Pomezia, Italy).

Results

Blood Biochemical Values

Levels of biochemical values are provided in Table 1. CRP ($P < 0.001$), ESR ($P < 0.001$), and leucocyte ($P < 0.05$) values were significantly higher in the active group than in the control group, whereas their values were significantly ($P < 0.05$ and $P < 0.001$) lower in the inactive groups than in the active group.

LP and Antioxidant Results

Values of LP, GSH, GSH-Px, and antioxidant vitamin values in neutrophil and serum of patients with BD are provided in Tables 2 and 3. In both patient and control groups, the LP, GSH, and GSH-Px and antioxidant vitamin values, as indicators of oxidative stress, were examined from the isolated neutrophil samples. In the active patient group, the serum LP level was higher in the active group than in the control group, although the GSH levels were lower in the active group than in the control group.

The LP levels in the serum ($P < 0.001$) and neutrophil ($P < 0.01$) from the patients were significantly higher in the active group compared to the inactive group and the control group. No statistically significant difference was determined in the LP levels between the healthy individuals and the inactive group, although serum and neutrophil LP levels were significantly ($P < 0.01$) lower in the inactive group than in the inactive group. The serum and neutrophil GSH levels of the healthy individuals was determined as significantly ($P < 0.01$) higher compared to the inactive and active group. No significant difference was determined in GSH levels between the active and inactive groups. No significant difference was also determined in GSH-Px levels in the control, active, and inactive groups. Serum vitamin A, vitamin E, and β-carotene concentrations were significantly ($P < 0.001$) lower in active patients than in the control group. Serum vitamin E and β-carotene concentrations were significantly ($P < 0.01$) higher in the inactive group than in the active group.

Table 1 Levels biochemical values in the control group and in patients with BD (mean ± SD, $n = 6$)

Parameter	Control	Patients (active)	Patients (inactive)
CRP (mg/ml)	5.20 ± 2.10	22.08 ± 23.24[b]	4.11 ± 2.78[d]
ESR (h)	2.82 ± 0.56	39.16 ± 14.97[b]	13.50 ± 6.53[d]
Leucocytes ($\times 10^3/mm^3$)	7.93 ± 1.81	9.50 ± 2.94[a]	6.95 ± 1.40[c]

[a] $P < 0.05$ and [b] $P < 0.001$ vs. control; [c] $P < 0.05$ and [d] $P < 0.001$ vs. active group

Table 2 Levels of LP, GSH, GSH-Px, and antioxidant vitamin values in neutrophil cells of control and patients with BD (mean ± SD, $n = 6$)

Parameter	Control	Patients (active)	Patients (inactive)
LP (µmol/g prot)	2.63 ± 0.37	3.67 ± 0.50[a]	2.06 ± 0.41[b]
GSH (µmol/ml)	5.51 ± 0.71	3.53 ± 0.53[a]	3.65 ± 0.51[a]
GSH-Px (IU/ml)	10.55 ± 1.43	10.59 ± 0.89	10.08 ± 1.44

[a] $P < 0.01$ vs. control; [b] $P < 0.01$ vs. active group

Table 3 Levels of LP, GSH, GSH-Px, and antioxidant vitamin values in serum of control and patients with BD (mean ± SD, $n = 6$)

Parameter	Control	Patients (active)	Patients (inactive)
LP (µmol/g prot)	2.27 ± 0.32	3.30 ± 0.29[b]	2.64 ± 0.38[c]
GSH (µmol/ml)	1.21 ± 0.38	0.76 ± 0.10[a]	0.90 ± 0.07[a]
GSH-Px (IU/ml)	2.14 ± 0.09	2.29 ± 0.15	2.27 ± 0.10
Vitamin A (µmol/l)	2.20 ± 0.18	1.74 ± 0.10[b]	1.69 ± 0.11[b]
Vitamin E (µmol/l)	12.80 ± 0.51	5.73 ± 0.67[b]	9.01 ± 0.57[a,c]
β-Carotene (µmol/l)	1.48 ± 0.23	0.62 ± 0.13[b]	0.93 ± 0.23[a,c]

[a] $P < 0.01$ and [b] $P < 0.001$ vs. control; [c] $P < 0.01$ vs. active group

Effects of Colchicine on Intracellular Ca^{2+} $[Ca^{2+}]_i$ Release in Neutrophil of Active Patients with BD

The effects of colchicine on intracellular Ca^{2+} $[Ca^{2+}]_i$ release in neutrophil are shown in Figs. 1 and 2. The

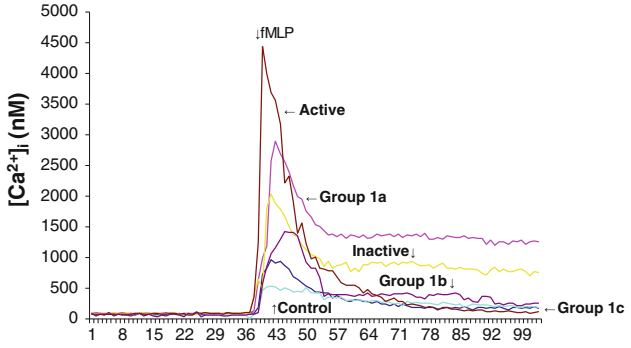

Fig. 1 Effects of colchicine (COL) treatments on intracellular Ca^{2+} release in their effects on intracellular $[Ca^{2+}]_i$ concentrations in neutrophil of control and active patients with BD. Stimulation was performed by fMLP. Group 1 ($n = 6$) comprised neutrophils from active BD patients, incubated with (group 1a, $n = 6$) colchicine, (group 1b, $n = 6$) diltiazem + verapamil, or (group 1c, $n = 6$) colchicine + diltiazem + verapamil. Inactive ($n = 6$) comprised neutrophils from inactive BD patients. The control group ($n = 6$) comprised neutrophils from healthy control subjects

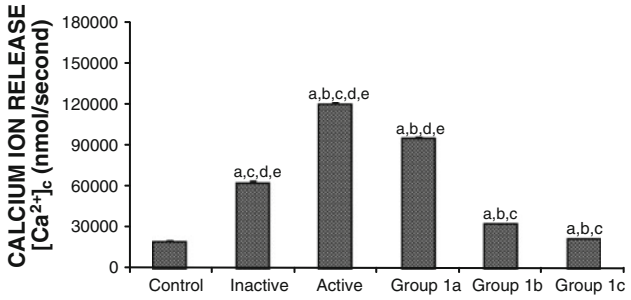

Fig. 2 Effects of colchicine treatments on intracellular Ca^{2+} release in their effects on intracellular $[Ca^{2+}]_i$ concentrations in neutrophil of control and active patients with BD. Group 1 ($n = 6$) comprised neutrophils from active BD patients, incubated with (group 1a, $n = 6$) colchicine, (group 1b, $n = 6$) diltiazem + verapamil, or (group 1c, $n = 6$) colchicine + diltiazem + verapamil. Inactive ($n = 6$) comprised neutrophils from inactive BD patients. The control group ($n = 6$) comprised neutrophils from healthy control subjects. $^{a}P < 0.01$ vs. control; $^{b}P < 0.01$ vs. inactive. $^{c}P < 0.01$ vs. colchicine. $^{d}P < 0.01$ vs. verapamil + diltiazem. $^{e}P < 0.01$ vs. colchicine + verapamil + diltiazem

release values of Ca^{2+} from the neutrophils into the cytosol were determined as significantly higher in the active group compared to the inactive group and the control group ($P < 0.01$). The release values of Ca^{2+} into the cytosol were determined as significantly higher in the inactive group compared to the control group ($P < 0.01$).

In the active BD subgroups, the release values of Ca^{2+} into the cytosol were determined as significantly low in group 1c (verapamil + diltiazem + colchicine) compared to group 1a (colchicine) and group 1b (verapamil + diltiazem) ($P < 0.01$). The release values of Ca^{2+} into the cytosol were determined as significantly low in group 1b (verapamil + diltiazem) compared to group 1a (colchicine) ($P < 0.01$). The release values of Ca^{2+} into the cytosol were determined as significantly low in group 1a (colchicine), group 1b (verapamil + diltiazem), and group 1c (verapamil + diltiazem + colchicine) compared to the basal calcium level of the active group ($P < 0.01$). The release values of Ca^{2+} into the cytosol were determined as significantly lower in group 1b (verapamil + diltiazem) compared to the inactive group ($P < 0.01$).

Discussion

Although it is known that there is increased neutrophil activation in BD, by what mechanism this neutrophil activation occurs is still unclear (Evereklioğlu 2005). This study aimed to explain the mechanisms of neutrophil activation that are involved in the etiopathogenesis of BD.

In inflammatory diseases, neutrophils are stimulated by fMLP to form the first barrier of defense against the attacking bacteria. By increasing the amount of intracellular Ca^{2+}, this stimulation increases free radical production. Increased ROS in the neutrophils causes DNA damage (Karaman et al. 2009). In a study by Şahin et al. (2011) of the role played by neutrophil activation in the pathogenesis of familial Mediterranean fever, a comparison was made of the measured levels of neutrophil $[Ca^{2+}]_i$ between a control group not receiving colchicine and patient groups experiencing attacks while receiving colchicine who had experienced remission and those who had not. Compared to the healthy control subjects, the patients in the group experiencing attacks were found to have a significantly high level of $[Ca^{2+}]_i$ (Şahin et al. 2011). Differing from the study by Şahin et al., in addition to active disease phase and healthy control subjects, measurements were also made of the basal Ca^{2+} levels of inactive phase BD patients. Thus, by measuring the changes in the neutrophil $[Ca^{2+}]_i$ concentration of both the active and inactive patient groups, the role of changes in the $[Ca^{2+}]_i$ concentration in the neutrophil in the etiopathogenesis of the disease was more clearly evaluated. The measurements of basal $[Ca^{2+}]_i$ concentration obtained from the patient groups were determined as significantly higher compared to the healthy individuals, and the active group basal $[Ca^{2+}]_i$ concentration measurements were significantly higher compared to the inactive group. Therefore, it is thought that an increase in neutrophil $[Ca^{2+}]_i$ concentration may have an effect on the disease etiopathogenesis and activation.

Because there is no specific treatment for BD, treatment is administered according to the system involved. Although the mechanism of the effect of colchicine on BD is not clearly understood, it has been shown to have an anti-inflammatory effect as an antioxidant and by inhibiting

leukocyte chemotaxis, and it is known that even at very low doses, adhesion of neutrophils to the endothelium and neutrophil migration decrease (Evereklioğlu 2005; Bréchard and Tschirhart 2008). Oral bioavailability varies between 25 and 50%. In the study by Şahin et al., the intracellular Ca^{2+} level of the patient group that had received colchicine and experienced remission was significantly low compared to the active attack patient group, and it was suggested that colchicine had an effect on Ca^{2+} release. In the current study, because we thought that colchicine may have an effect on neutrophil $[Ca^{2+}]_i$ concentration variations in BD pathogenesis and activation, we incubated neutrophils with colchicine in vitro. Thus, our aim was to eliminate the bioavailability differences, unlike the study by Şahin et al. (2011).

After incubation with colchicine, the measurements of the neutrophil $[Ca^{2+}]_i$ concentration of the active group were determined to be significantly low compared with the basal measurements, showing that colchicine had decreased the neutrophil $[Ca^{2+}]_i$ concentration in the patients with BD. Because it was not known through which Ca^{2+} channel the colchicine had an effect on the Ca^{2+}, the voltage-sensitive Ca^{2+} gates were blocked with the calcium channel blockers of diltiazem or verapamil (Shima et al. 2008).

Thus, the aim was to establish through which channel colchicine had an effect on the intracellular calcium level in the neutrophils. The neutrophils taken from active patients with BD were incubated; one section with colchicine, one section had voltage-dependent channels blocked with verapamil + diltiazem, and another section with colchicine + verapamil + diltiazem. A decrease of a significant level in the $[Ca^{2+}]_i$ concentration was determined in the active patient neutrophils incubated with colchicine + verapamil + diltiazem compared to those incubated with verapamil + diltiazem. These results suggest that colchicine had made the Ca^{2+} signal in the neutrophils in BD through a channel other than the voltage-dependent Ca^{2+} channels. We observed in this study that neutrophils from active patients with BD that were incubated with verapamil + diltiazem showed a definite rate of decrease in $[Ca^{2+}]_i$ concentration compared to neutrophils incubated with colchicine, which suggests a need to investigate the therapeutic use of the voltage-dependent calcium channel blockers verapamil and diltiazem for BD.

In the current study, vitamin A, vitamin E, and β-carotene concentrations were lower in the active group than in the control or inactive groups. In patients with BD, there is an imbalance between the oxidant and antioxidant systems, and with increased neutrophil functions, ROS production increases (Yazici et al. 2004; Evereklioğlu 2005), while vitamin A, vitamin E, and β-carotene concentrations in plasma were lower in patient group than in the control subjects (Kökçam and Nazıroglu 2002). ROS released from active neutrophils and increased lipid peroxide products are responsible for BD endothelial dysfunction. In a study in which endothelial functions were evaluated by measuring the brachial artery flow in patients with BD, the endothelial dysfunction improved rapidly by giving vitamin C, which has antioxidant properties, thus showing that oxidative stress plays a role in the disease etiopathogenesis (Köse et al. 1995; Chambers et al. 2001; Kökçam and Nazıroglu 2002).

The blood of patients with BD has conflicting GSH and antioxidant enzyme results. In a study by Taysi et al. (2002), serum copper, zinc, superoxide dismutase, and GSH-Px activity with active and inactive ocular involvement and nonocular involvement was shown to be increased to a significant degree in patients with BD compared to the control group. In another study, the superoxide dismutase level had decreased in patients with BD compared to the control group, and no change was observed in GSH-Px activity (Köse et al. 1995). In a study by Sandıkci et al. (2003), the plasma LP (as malondialdehyde) levels of active patients with BD compared to the inactive and control groups were observed to be significantly high, whereas the GSH-Px level was significantly low. From these results, it can be said that antioxidant activity is decreased by the disease activation. Kökçam and Nazıroğlu (2002) also reported that catalase, GSH-Px activities, and GSH levels in erythrocytes and plasma of patients with active BD were lower in the patient group than in control subjects, while they were found to be higher in the vitamin E treatment group than in the patient group. In the current study, LP levels were higher in the active group than in the control group, although GSH levels were decreased in the active group. However, GSH-Px activity did not change in the three groups. The results were confirmed by results of Köse et al. (1995), Kökçam and Nazıroğlu (2002), and Sandıkci et al. (2003).

Although oxidative stress of neutrophils has a role in patients with BD, precisely how oxidative stress plays a role in the pathogenesis of patients with BD is not known (Yazici et al. 2004; Evereklioğlu 2005). Unlike other studies, it was thought that more robust results would be obtained from the current study by examining, as indicators of oxidative stress, the LP and antioxidant enzyme levels in neutrophils, which play an important role in the disease pathogenesis, rather than in serum and erythrocytes, which can be affected by many factors. Because neutrophils are nucleated cells, when there is oxidative stress in the nucleus and an increase in intracellular calcium leading to DNA damage, they may thus play a role in neutrophil activation (Ayub and Hallett 2004; Karaman et al. 2009). In the current study, the LP levels of the active patient group were found to be significantly high compared to the

inactive patient group and the control group. Because there was no significant difference in the LP levels between the inactive patient group and the control group, it is thought that the increase in oxidative stress may be responsible for BD activation.

The GSH levels of the active and inactive patients with BD were found to be significantly low compared to the control group, and because no significant difference was determined in the GSH levels between the active and inactive patients with BD, this supports the notion that the imbalance between oxidative stress and the antioxidant system plays a role in the disease pathogenesis. No significant difference was determined in the GSH-Px levels between the groups. These results of the current study are similar to those of Gurbuz et al., who reported that although an increase was found in the LP levels of patients with familial Mediterranean fever, no difference was observed in GSH-Px levels (Gurbuz et al. 2005). It is known that GSH-Px and catalase convert hydrogen peroxide to water in the same way. In this study, that fact that there was increased LP levels in the neutrophils and no increase in the GSH-Px levels suggests that hydrogen peroxide was broken down by catalase. Therefore, there is a need for research into catalase levels in the neutrophils of patients with BD.

This study is important because to our knowledge, it is the first to examine the effects of Ca^{2+} signaling, oxidative stress parameters, and the level of colchicine molecules on the Ca^{2+} mechanism in the neutrophils of patients with BD. Because increased oxidative stress and neutrophil Ca^{2+} entering into the cytosol has been observed particularly in the active phase of BD, and because oxidative stress and release of neutrophil Ca^{2+} into the cytosol have been determined to have increased, especially in the activation phase, it is thought that the neutrophils playing a role in BD etiopathogenesis are activated by oxidative stress caused by Ca^{2+} release into the cytosol. One of the significant results obtained from this study is that colchicine used in BD treatment led to a reduction in cytoplasmic Ca^{2+} release in the neutrophils. It is thought that colchicine implements the neutrophil Ca^{2+} release by a Ca^{2+} channel other than the voltage-dependent Ca^{2+} channels. Thus, future studies may be able to develop new treatment approaches by targeting voltage-dependent calcium channels with the substantial reduction from verapamil and diltiazem observed in intracytoplasmic Ca^{2+} levels in the neutrophils of patients with BD.

In conclusion, because there is increased oxidative stress and Ca^{2+} release into the neutrophil cytosol in patients with BD, this increase is more evident during the disease activation phase. Colchicine used in BD treatment leads to a reduction in cytoplasmic Ca^{2+} release in the neutrophils. There is now thought to be the possibility that the colchicine Ca^{2+} signal in the neutrophils is made through the voltage-dependent Ca^{2+} channels. In patients with BD, verapamil and diltiazem reduce the intracytoplasmic Ca^{2+} level in the neutrophils via voltage-dependent channels. Future studies targeting voltage-dependent calcium channels may have implications for new treatment approaches.

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