The Effect of Colchicine on Chemiluminometric Evaluation of Leukocyte Function in Behcet's Disease Patients

Kolşisinin Behçet Hastalarında Lökosit Fonksiyonları Üzerindeki Etkisinin Kemiluminometrik Olarak Değerlendirilmesi

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ABSTRACT Objective: The aim of the present study is to investigate the interaction of colchicine with the peak chemiluminescence (CL) signal of the cell free systems and stimulated leukocytes of healthy volunteers or patients with Behcet's disease. **Material and Methods:** Luminol-enhanced chemiluminescence method was used in the experiments. In cell-free experiments, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), hypochlorous acid ($HOCl^-$), superoxide ($O2^-$) and peroxynitrit (ONOO-)–induced chemiluminescence responses were initiated by hydrogen peroxide (16 mM), $FeSO_4$ (50 nM), NaOCl (5 mM), xanthine (X) (0.1mM) with xanthine oxidase (XO) (20 mU/mL) and peroxynitrite, respectively, in the presence of luminal (250 μM) in physiologic buffer saline (PBS) at 37°C. **Results:** Colchicine inhibited the peak CL signals generated by H_2O_2 , OH^-, O_2- and $HOCl^-$ or $ONOO^-$. Human leukocytes from healthy or diseased subjects stimulated by a chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP) ($4x10^{-6}$ M) induced CL signal which was also inhibited by colchicine. **Conclusion:** Results suggested that responsible from the inhibitory effect of colchicine on the peak CL of FMLP-stimulated healthy or Behcet's diseased leukocytes might be result of free radical scavenger effect of colchicine.

Key Words: Behcet syndrome; colchicine; chemiluminescence measurements; antioxidants

ÖZET Amaç: Bu çalışmanın amacı, sağlıklı gönüllülerde ve Behçet hastalığı olan kişilerde, hücresiz sistemlerin ve uyarılmış lökositlerin tepe kemiluminesens (CL) sinyalleri ile kolşisin arasındaki etkileşimi araştırmaktır. **Gereç ve Yöntemler:** Deneylerde luminol-bazlı CL yöntemi kullanılmıştır. Hücresiz deneylerde hidrojen peroksit (H_2O_2), hidroksil radikali (OH^-) hipokloröz asit ($HOCl^-$), süperoksit ($O2^-$) ve peroksinitrit ($ONOO^-$) tarafından uyarılmış CL cevaplar 37 °C'de fizyolojik tampon çözeltisinde (PBS) luminal'in (250 μM) varlığında hidrojen peroksit (16 mM), $FeSO_4$ (50 nM), NaOCl (5 mM), ksantin (X) (0.1 mM)'dan oluşan ksantin oksidaz (XO) (20 mU/mL) ve peroksinitrit kaynaklı CL sinyali ölçümüyle değerlendirilmiştir. **Bulgular:** Kolşisin hücresiz sistemde H_20_2, OH^-, $O2^-$ ve $HOCl^-$ veya $ONOO^-$ ile oluşturulan CL tepe sinyallerini inhibe etmiştir. Ayrıca bir kemotaktik peptid olan "N-formil-metionik-lösil fenilalanin (FMLP)" ($4x10^{-6}$ M) ile uyarılmış gönüllü veya hasta kişilerin lökositlerinde oluşan CL sinyalleri de kolşisin tarafından inhibe edilmiştir. **Sonuç:** Bu sonuçlar, kolşisinin sağlıklı gönüllülerden ve Behçet sendromlu hastalardan izole edilip FMLP ile stimüle edilen lökositlerin CL piki üzerindeki inhibitör etkisinden, kolşisinin serbest radikal süpürücü etkisinin sorumlu olabileceğini göstermektedir.

Anahtar Kelimeler: Behçet sendromu; kolsişin; kimyasal flöresansin ölçümleri; antioksidanlar

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During the respiratory burst, stimulated polymorphonuclear leukocytes generate superoxide (O_2^-) radical. This is followed by H_2O_2, OH^- and, $HOCl^-$ production.[1,2] Free radicals are shown to be in-

volved in a wide range of diseases such as ischemia-reperfusion injury, atherosclerosis, lung injury, sepsis, arthritis and chronic inflammatory disorders such as romatoid arthritis (RA) and Behcet's disease (BD). Behcet's disease is a multisystem disease characterized by recurrent oral and genital ulcers, relapsing uveitis, mucocutaneous, articular, urogenital, vascular, intestinal, pulmonary and neurologic manifestations. The pathogenic aspects of the disease pointed to significant oxidative stress in patients with BD, the severity of which may arise from impaired antioxidant mechanisms.[3] The functions of polymorphonuclear leukocytes (PMN) such as chemotaxis, phagocytosis and O_2^- generation have been shown to be increased in BD. Stimulated inflammatory cells represent a major source of oxygen radicals and metabolites. Leukocytes, which are activated by a variety of stimuli, produce a series of reactive oxygen species. The initial product of oxygen reduction is superoxide, generated during a respiratory burst. Following superoxide generation, other oxygen metabolites may then be formed including H_2O_2, singlet oxygen, hydroxyl radical and HOCl. These metabolites are being formed in a reaction catalyzed by myeloperoxidase.[2,4] Another free radical, nitric oxide, has been demonstrated to be released from leukocytes on activation by different stimuli. It has been shown that superoxide radical can react with nitric oxide to form the potent oxidant peroxynitrite. The peroxynitrite anion is rapidly protonated to peroxynitrous acid which the half-life of about one second at pH 7.4.[4,5] Peroxynitrous acid decomposes rapidly to nitrate and can serve as a precursor for other potent reactive species, including nitrogen dioxide, nitronium ion and an intermediate with hydroxyl radical-like reactivity towards a variety of biological molecules. Free radical scavengers or antioxidants are also found to be beneficial in shock, inflammation, and ischemia-reperfusion where free radicals are believed to be involved.[6]

Chemilumines cence (CL) has been widely used as a sensitive assay for monitoring free radicals and reactive metabolites released from cell, organ systems and enzymes.[7,8] Generation of reactive oxygen species and metabolites emits light which can be monitored by a variety of luminometers.[9] Light emission can be markedly amplified by luminol which measures a mixture of free radicals.[9,10] In the present study, the chemiluminescence method was used for assessment of the scavenging activity of colchicine against free radicals or metabolites generated by either stimulated human leukocytes or cell-free systems.

Colchicine interacts with the intracellular microtubuli, thus interfering with intracellular granula transport and secretion of mediators.[11] It inhibits leukocyte chemotaxis at rather low concentrations and alters expression of adhesion molecules on the surfaces of neutrophils and their potential to produce cytokines. Colchicine, an inhibitor of phagocytosis is a potent drug in treating Behcet's disease which free radicals including superoxide (O_2^-) are probably involved in inflammatory manifestations of the disease.[12]

The aim of the present study is; to investigate the interaction of colchicine and reactive oxygen species (OH^- , O_2^- , H_2O_2, $HOCl^-$ and, $OONO^-$)-induced chemiluminescence in the cell-free systems; to examine the effect of colchicine on luminol-dependent chemiluminescence signal generated by stimulated leukocytes from healthy subjects or patients with Behcet's disease.

MATERIAL AND METHODS

CL GENERATED FROM HEALTHY OR DISEASED LEUKOCYTES

Patients

Human venous blood was obtained from healthy volunteers (n=10) and patients with BD (n=11; 3 of them were not given any treatment) followed up at Ankara University Multidisciplinary Behcet's Disease Centre. Every subject gave informed consent, all procedures were in accordance with the Helsinki Declaration and the study was approved by the Multidisciplinary Behcet's Disease Center of University of Ankara. All the patients were non-smoker and untreated group (n= 3) was just diagnosed (diagnosis was conformed to the criteria of International Study Group for Behcet's disease) and

having none treatment. Treated group (n= 8) was taking only colchicine (0.5-1.5 mg, p.o., daily).

Isolation and Separation of Leukocytes

Leukocytes were isolated from volunteer's blood.[13] Blood (9 mL) was taken into tubes containing 3.8% sodium citrate (1 mL). Dextran was added and allowed to sediment at room temperature for 60 min. The leukocyte rich supernatant was removed and centrifuged at 900 r.p.m. for 20 min. Erythrocyte lysis was performed by washing cells with 0.2% NaCl for 30s and mixing immediately with double volume of 1.6% NaCl. Then, leukocytes were centrifuged at 900 r.p.m. for 15 min and the pellet was resuspended in Hank's buffered salt solution (HBSS) containing 1 mM calcium (pH 7.4). Leukocytes were washed with HBSS three times. After leukocyte count in a cell counter (Contraves, Digicell 300, Zurich, Switzerland), cell yield was adjusted to 10^7 cells ml^{-1} (stock cell suspension) by adding HBSS. The stock cell suspension was stored at room temperature until use. Duplicate assays were performed in all experiments.

FMLP-Induced CL From Healthy or Diseased Human Leukocytes

Stock leukocyte cell suspension (0.1 mL) was diluted with HBSS in a cuvette (total volume of 1.0 ml) and 20 µl luminol (50 µM; final cuvette concentration) was added, producing a final cell yield of 10^6 cells ml^{-1}. After then by adding FMLP ($4x10^{-6}$ M), luminol-CL was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The CL produced was measured continuously and recorded on a computer by using the Luminometer 1250 programme (version 1.12, BioOrbit) for 10 min.

After recording the peak CL of FMLP-stimulated leukocytes experiments were repeated in the presence of colchicine at various concentrations (10^{-5}-10^{-2} M). These concentrations had chosen within the theurapeutic concentrations that calculated by the time-concentration curve of colchicine.[14]

CL-Generated By Cell-Free Systems

Luminol chemiluminescence is an accepted method for assessing PMN's oxidant activity and effects of colchicines on leucocytesb. In invitro part of the study, the baseline data are assessed initially then, cell-free systems used to generate various reactive oxygen species and potent cytotoxic anions were described below:

Ferrous Iron-Induced CL

Hydroxyl radicals were generated by the addition of ferrous iron (Fe^{+2}) to the buffer solution, as described and modified previously.[15,16] Ferrous iron reduces molecular oxygen to superoxide radical, which in turn dismutases to H_2O_2. Further reduction of H_2O_2 by Fe^{+2} produces OH^- radical. Freshly prepared $FeSO_4$ (50 nM in 0.9% NaCl) was added to physiologic buffer saline (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) plus luminol (250 µM, prepared daily in 2M NaOH and diluted with PBS) mixture in the chemiluminometer cuvette and then CL was recorded only for 3 min.

H_2O_2-Induced CL

16 mM H_2O_2 was added to the buffered solution plus luminol (250 µM) mixture in the chemiluminometer cuvette. CL was recorded continuously for 10 min.

Xanthine (X) + Xanthine Oxidase (XO)-Induced CL

Xanthine-xanthine oxidase (X-XO) system is used to generate O_2^- and H_2O_2 enzymatically [8]. 0.1 mM xanthine was added to the PBS plus luminol (250 µM) mixture in the chemiluminometer cuvette and after the addition of 20 mU/ml XO, generated CL at 37°C was recorded continuously for 10 min.

HOCl-Induced CL

HOCl was prepared as previously described by Vissers et al.[17] To obtain HOCl and OCl^- mixture (1:1), NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4 immediately before the CL measurement. HOCl (5 mM) was injected in to the PBS and luminol (250 µM) mixture in the chemiluminometer cuvette and CL was measured continuously for 3 min.

Peroxynitrite Synthesis and Induced CL

Peroxynitrite was prepared by using a quenched-flow reaction as described previously.[18] Briefly, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M H_2O_2 containing 0.7 M HCl and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H_2O_2 was removed by addition of manganese dioxide (MnO_2) powder to the peroxynitrite solution. The mixture was shaken for 5 min and then MnO_2 was removed by passage over a cellulose acetate disposable filter. The solution was used freshly or frozen at –20°C for as long as a week. The final concentration of peroxynitrite was determined spectrophotometrically in 1.2 M NaOH (e_{302}=1670 M^{-1} cm^{-1}). Dilutions of this peroxynitrit stock solution were made in 1.2 M NaOH with the final dilution in 0.1 M NaOH before use.

Catalase (50 U/ml) was added into PBS-luminol (250 μM) mixture to remove H_2O_2 left after MnO_2 and after the injection of $ONOO^-$ (20 nM) into the chemiluminometer cuvette, CL was measured continuously for 3 min.

Drugs

Colchicine was a gift from İbrahim Ethem Ulagay Drug Company (Turkey). Luminol sodium, ferrous sulfate heptahidrate, catalase (from bovine liver), sodium hypochloride, N-formyl-methionyl-leucyl-phenylalanine (FMLP) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) and H_2O_2 from Merck (Darmstadt, Germany).

Experimental Protocol and Statistics

The effect of the colchicine (10^{-5}-10^{-2} M) on the peak CL were examined by addition to the mixture before the stimulant. Duplicate assays were performed in all experiments. Results were calculated as peak CL or a % of the peak CL and expressed as mean ± S.E.M. n refers to the number of individual volunteers (for leukocyte experiment) and number of experiments (for cell-free assays). Analysis of variance (ANOVA) was used to assess the observed differences in CL between concentrations and Student-Newman-Keuls used as a post hoc test. P<0.05 was considered statistically significant.

RESULTS

FMLP ($4x10^{-6}$ M) produced 4383 ± 224.1 mV (n=21) CL signal in the leukocyte assay from healthy volunteers (Figure 1A) and 2039 ± 127.4 mV (n=10) from untreated Behcet's disease patient. FMLP-induced response was depressed by colchicine in a concentration-dependent manner. The most marked reduction of the FMLP-induced luminol CL was observed with colchicine (Figure 1B). A significant decrease in the presence of colchicine in FMLP-induced luminol CL was recorded at 1 mM (18+3%, n=6), at the concentrations tested, the maximum reduction with colchicine was observed at 10 mM (96 + 0.1%, n=7).

H_2O_2 (16 mM), $FeSO_4$ (50 nM), HOCl (5 mM) and $ONOO^-$ (20 nM) produced 3624 ± 335 mV (n=26), 3310 ± 245 mV (n=13), 5305 ± 238 mV (n=7) and 4245 ± 486 mV (n=11) luminol-CL peaks, respectively. These values were comparable to those obtained by FMLP ($4x10^{-6}$ M) (4383 ± 224 mV) (n=21) stimulation in the experiments with leukocytes. X (0.1 mM) and XO (20 mU/ml) induced peak CL signal was 371 ± 21 mV (n=9).

EFFECTS OF COLCHICINE ON LUMINOL CL IN FMLP-STIMULATED HEALTHY OR DISEASED LEUKOCYTES

Leukocyte number in 1.0 mL volume of blood was 16000 ± 1000 in healthy volunteers and 35000 ± 3300 in patients with BD and 39000 ± 3000 in

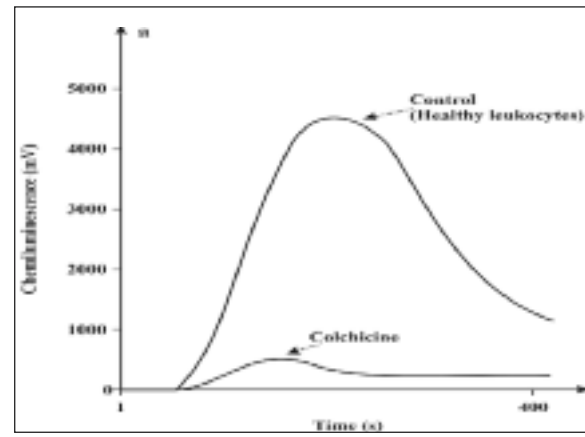

FIGURE 1A: Typical traces representing the effects of colchicine at 10-2M on FMLP (4x10-6 M) stimulated luminal-enhanced chemiluminescence in human isolated leukocytes .

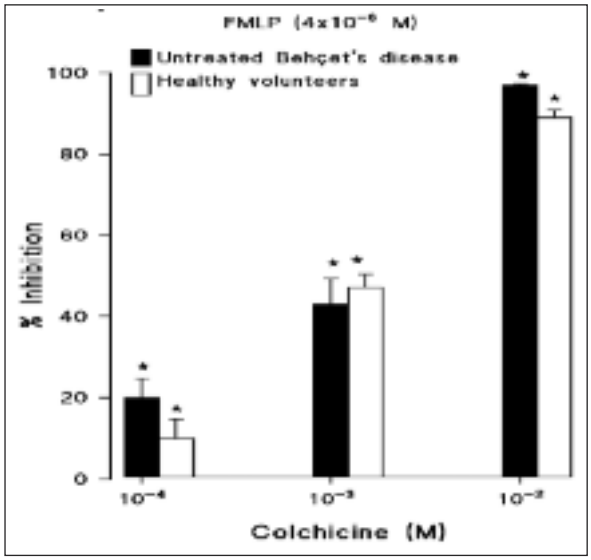

FIGURE 1B: Concentration-dependent effects of colchicine (10-4-10-2M) (n= 6-17) on FMLP-stimulated leukocytes of healthy or patient with Behcet's disease .Data are shown as mean ± S.E.M. *P<0.05 significantly less than its control value.

colchicine-treated patients (Table 1). FMLP ($4x10^{-6}$ M) produced 4383 ± 224.1 mV (n=21) CL signal in the healthy leukocytes. However, in the leukocytes obtained from untreated patients with BD, FMLP ($4x10^{-6}$ M) produced significantly lower CL values (2039 ± 127.4 mV) (n=19) than those obtained from healthy leukocytes ($p< 0.05$).

In the leukocytes obtained from colchicine treated patients with BD, FMLP ($4x10^{-6}$ M) induced very smaller (88 ± 25.8 mV) CL values (n=23) (Table 1) than those of untreated patients or healthy volunteers.

Colchicine inhibited FMLP-induced CL signal in a concentration dependent manner in both healthy and diseased leukocytes (from untreated BD) (Figure 1). Inhibition was statistically significant in all of the used concentrations of colchicine (10.0 ± 4.8 and 20.0 ± 4.6 % at 10^{-4} M, 47.0 ± 3.4 and 43.0 ± 6.3 % at 10^{-3} M and, 89.0 ± 1.9 and 97.0 ± 0.3 % at 10^{-2} M concentrations, respectively), (n=6-17).

EFFECT OF COLCHICINE ON FERROUS IRON-INDUCED CL

Inhibition of colchicine on $FeSO_4$-induced luminol-CL was significant and concentration dependent (1.0 ± 2.3% at 10^{-5} M, 31.0 ± 2.5 % at 10^{-4} M, 71.0 ± 0.8 at 10^{-3} M and 89.0 ± 2.1 % at 10^{-2} M concentrations) (n=6-7) (Figure 2).

EFFECT OF COLCHICINE ON H_2O_2-INDUCED CL

Inhibition of colchicine on H_2O_2-induced luminol-CL was also found significant and concentration dependent (7.0 ± 4.1 % at 10^{-5} M, 31.0 ± 5.5 at 10^{-4} M, 81.0 ± 0.7 % at 10^{-3} M and 94.0 ± 1.6 % at 10^{-2} M concentrations) (n=6-11) (Figure 2).

EFFECT OF COLCHICINE ON XANTHINE (X)+ XANTHINE OXIDASE (XO)-INDUCED CL

Colchicine produced significant inhibition on X+XO-induced luminol-CL (12.0 ± 4.8 % at 10^{-4} M, 55.0 ± 2.6 % at 10^{-3} M and, 86.0 ± 0.3 % at 10^{-2} M concentrations) (n=5-10) (Figure 3).

EFFECT OF COLCHICINE ON HOCL-INDUCED CL

$HOCl^-$ -induced luminol-CL was inhibited by colchicine concentration dependently (3.0 ± 1.0 % at 10^{-4} M, 24.0 ± 0.9 % at 10^{-3} M and, 60.0 ± 1.2 % at 10^{-2} M concentrations) (n=6-10). (Figure 3).

EFFECT OF COLCHICINE ON PEROXYNITRITE-INDUCED CL

Colchicine-induced inhibition of $ONOO^-$-induced luminol-CL was also significant and concentration dependent (1.0 ± 1.7% at 10^{-4} M, 73.0 ± 2.8% at 10^{-3} M and, 99.0 ± 0.1 % at 10^{-2} M concentrations) (n= 6-8) (Figure 3).

TABLE 1: Leukocyte numbers of healthy and diseased (treated or untreated) patients and FMLP ($4x10^{-6}$)-stimulated peak CL values of leukocytes (10^6 cell/mL).

Groups	n	Leukocyte number/mL	n	Peak CL signal (mV) of isolated leukocytes
Healthy volunteers	10	16000 ± 1000	21	4383 ± 224.1
Untreated Patients	3	39000 ± 3000*	10	2039 ± 127.4*
Treated Patients	8	35000 ± 3300*	23	88 ± 25.8*

n: Number of experiment; * p<0.05 compared to healthy volunteers.

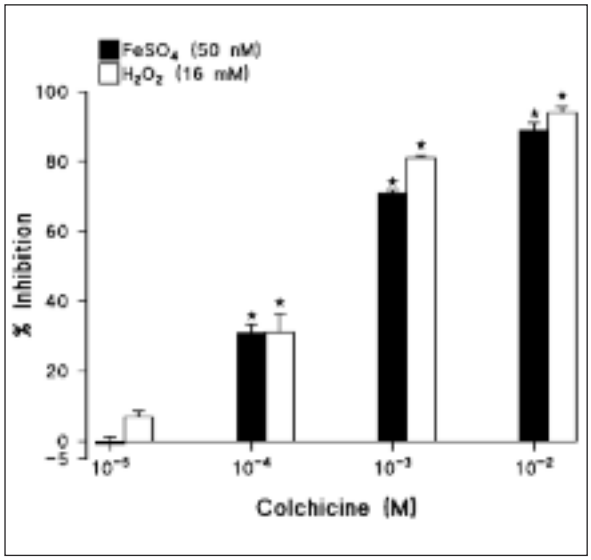

FIGURE 2: Inhibitor effect of colchicine (10^{-5}-10^{-2} M) on luminol CL stimulated by FeSO4 ($5x10^{-8}$ M) (n=6-10) or H2O2 ($1.6x10^{-2}$ M) (n=6-11). Data are shown as mean ± S.E.M. *P<0.05 significantly less than its control value.

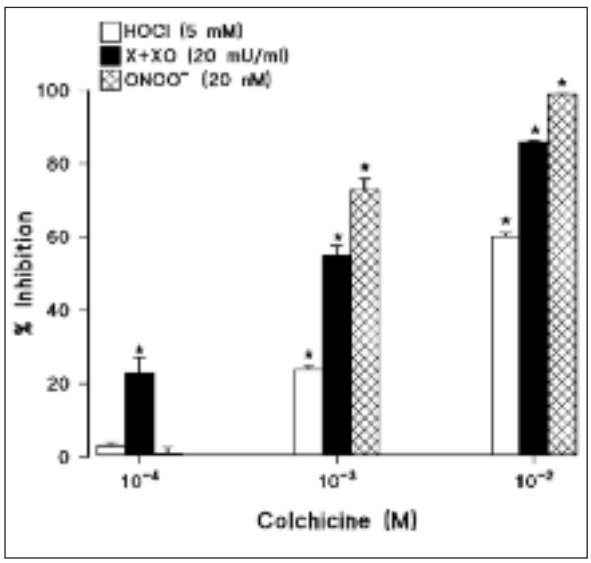

FIGURE 3: Inhibitor effect of colchicine (10^{-4}-10^{-2} M) on luminol CL stimulated by HOCl ($5x10^{-3}$ M) (n=6-7), X+XO (20mU/mL) (n=5-10) or ONOO- ($2x10^{-8}$ M) (n=6-8). Data are shown as mean ± S.E.M. *P<0.05 significantly less than its control value.

Colchicine-induced inhibition was prominent on $ONOO^-$, $FeSO_4$ or H_2O_2-induced peak CLs and was fair on $HOCl^-$- induced peak CL.

DISCUSSION

NADPH-oxidase is an enzyme that responsible from the production of superoxide radical. FMLP stimulates NADPH-oxidase by binding its specific membrane reseptor on phagocytes. In the present study, leukocyte numbers were found significantly higher in patients with Behcet's disease compared to those of healthy volunteers in a 1 ml blood (Table 1) as generally expected in all inflammatory diseases.[19] Neutrophil infiltration is a characteristic finding observed in skin and eye lesions of patients with Behcet's disease and hyperfunction of peripheral blood neutrophils from such patients has been noted.[20] Neutrophils isolated from patients with BD produced more superoxide radical than those from healthy subjects. Enhanced super oxide generation and decreased superoxide scavenger activity of peripheral blood leukocytes in BD were reported previously and it has been suggested that this was due to primary dysfunction of neutrophils or to be secondary to bioactive substances in the serum.[21,22] 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazol (1,2-a) pyrazin-3-one (MCLA) were used for dependent CL technique. MCLA was a CL probe which can respond to singlet oxygen as well as superoxide. In the present study, FMLP-stimulated CL values (as a reflection index of leukocyte ability to generate free radicals for defensive action against microorganisms) of fixed amount of leukocytes (10^6 cell in 1 ml) were significantly lower than those of control group (Table 1).

As a result, colchicine inhibited some aspects of polymorphonuclear leukocyte activity. The required concentrations were significantly in the therapeutic range that effectively treated patients.[23]

In the present study we have shown for the first time that colchicine significantly inhibited the luminol enhanced-CL in both cell-free systems and leukocytes obtained from healthy volunteers or patients with BD. Among the cell-free systems examined, CL signal generated by the X+XO system, was also inhibited by colchicine, demonstrating a direct superoxide scavenger activity. It has been shown that superoxide radical can react with nitric oxide to form potent oxidant peroxynitrite.[4] In addition, $ONOO^-$-induced luminol CL was most markedly decreased by colchicine. Ability of colchicine to scavenge both O_2^- and $ONOO^-$ may be involved in its potent therapeutic effect in cer-

tain inflammatory diseases. Phagocytes defense against microorganism by the production of superoxide and O_2^- which secondarily generates HOCl from H_2O_2 and chloride ions by a reaction catalyzed by the myeloperoxidase; an enyzme present in neutrophils.[24]

Present study also showed that colchicine was effective as a scavenger of H_2O_2, although it slightly reduced HOCl-induced luminol CL in cell-free systems. Colchicine-induced inhibition of CL signal produced by OH^- radical generating system in cell-free experiments also showed that colchicine has a potent radical scavenger effect, since OH^- is a potent cytotoxic radical and can play a role in the pathogenesis of several disease states.[25]

In FMLP-stimulated leukocyte experiments, colchicine inhibited luminol-CLs effectively, although it has a slight inhibitor effect on HOCl-induced CL signal in cell-free experiments. This might represent that the predominance of one reactive oxygen species to the others released from leukocytes on activation, depends on the stimulant applied and the species examined. In addition, colchicine induced inhibition of chemiluminescence is partly due to direct interaction between the antioxidant and oxidant, and partly due to interference of the antioxidant with the luminol-oxidant chemiluminescence reaction.[26-28]

Colchicine produced a direct free radical scavenger effect in either cell-free or leukocyte experiments and this effect can involve in the anti-inflammatory effects of colchicine in therapeutic doses and diverse pathological states.

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