Nitric oxide and superoxide dismutase in rheumatoid arthritis: Correlation with disease activity

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Abstract

Aim: To analyse serum and synovial fluid nitric oxide (NO) concentrations and erythrocyte CuZn superoxide dismutase (SOD) activity in rheumatoid arthritis (RA) patients and to determine the importance of imflammatory markers in reflect disease activity.

Methods: 20 RA patients and 15 healthy controls were included. NO level was determined spectrophotometrically by the Griess reaction and CuZn SOD activity by the method of Sun et al.

Results: Erythrocyte sedimentation rate (ESR), C-reactive protein and leukocyte levels were determined as inflammatory markers. Significantly higher ESR ($p<0.001$) and leukocyte levels ($p<0.01$) and serum and synovial fluid NO levels ($p<0.01$, $p<0.05$) and lower CuZn SOD activity ($p<0.05$) were observed in RA patients. Classification of RA patients according to disease activity score (DAS) revealed significantly higher synovial fluid NO level ($p<0.05$) and lower CuZn SOD activity ($p<0.05$) in RA patients with DAS≥2.7.

Conclusions: Increased serum and synovial fluid NO levels and decreased CuZn SOD activity up-regulated inflammatory response are observed in RA patients.

Keywords: nitric oxide, superoxide dismutase, rheumatoid arthritis, imflammatory markers, synovial fluid

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Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by synovial fluid inflammation involving peripheral joints, cartilage destruction, bone erosion and subsequent joint deformity[1].

Numerous studies carried out on inflammatory diseases such as RA revealed increased endogenous NO synthesis due to the activation of iNOS pathway[2-4]. A number of cells within the joint, including endothelial cells of synovial capillaries, mesenchymal cells, neutrophils, fibroblasts, lymphocytes, mast cells and macrophages are able to generate substantial quantities of NO. Synoviocytic fibroblasts seem to be the main source of NO in rheumatoid synovium [5].

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Over production of NO, as a mediator of inflammation and/or tissue destruction ,may be important in the pathogenesis of RA. As NO is highly labile, measurement of the relatively stabile metabolites, nitrate and nitrite, (NO_x), is employed as an index of NO production and as a marker of NOS activity.

Increased NO in articular cartilage can result from increased pro-inflammatory cytokines like IL-1 and TNF-α. TNF-α overproduction is thought to be the main contributor to increased ROS release in patients with RA .TNF-α ,not only causes cell damage, but also inhibits CuZn SOD activity (SOD_1 and SOD_3) [3-6,7]. SOD activity is a key component of the cellular antioxidant armamentarium that protects cells and extracellular matrix from the harmful effects of O_2^- and its derivatives.

In this study; we aimed to measure serum and synovial fluid NO concentrations and erythrocyte CuZn SOD activity in patients with RA and compare them with healthy controls. Furthermore we also investigated the correlations between NO levels and SOD activity and laboratory paramaters of disease activity in RA.

2. Patients and methods

Patients

This study was conducted in 20 patients attending Rheumatology Department of Internal Medicine at Cerrahpaşa Medical Faculty, Istanbul University. The patients fulfilled the 1987 revised American College of Rheumatology criteria for RA [8]. The study protocol was approved by the Ethics Committee of Cerrahpaşa Medical Faculty. Written informed consent was obtained from all patients and controls. A total of 20 patients with RA (13 females, 7 males, mean age 40.20 ± 13.80) were included in the study. There were two control groups. 15 healthy age matched individuals (8 females, 7 males, mean age 37.06 ± 9.73) formed the control group for blood samples. Ten age matched individuals (4 females, 6 males, mean age (36.50 ± 8.10) with acute knee injury were recruited from Orthopedics and Traumatology Department to form the control group for synovial fluid analyses.

Methods

Disease Activity Score (DAS), an established measure for the evaluation of current RA activity, was determined [9]. Relevant parameters of the score include the following: total number of tender joints of 53 joints [Ritchie Articular Index (RAI)], swollen joint count of 44 joints (TSJI), erythrocyte sedimentation rate (Westergren method, mm/h), and general health self-assessment (GHA) by marking a 100-mm visual analog scale (VAS).

The DAS was calculated using the following formula:

DAS = 0.53938 x √RAI + 0.06465 x TSJI + 0.33 x LnESR + 0.00722 X GHA

The RA group was sub-grouped according to disease activity: moderate RA activity (DAS > 2.7, n = 13), low RA activity (DAS < 2.7, n = 7). The patients were newly diagnosed, and thus were undergoing only nonsteroidal anti-inflammatory drug (NSAID) treatment.

Samples

Venous blood samples from patients and controls were collected between 8 and 9 AM after an overnight fast, and sera were stored at -70°C until the analysis. Synovial fluid samples from RA patients and from nonarthritic control (NAC) group were obtained from knee joints by arthrocentesis, aseptically aspirated and transmitted into heparinized tubes, centrifuged at 5,000 x g for 10 min and stored at -70°C until analyzed.

Determination of nitric oxide level

Plasma nitric oxide (NO) was measured as its stable metabolites nitrate (NO_3) and nitrite (NO_2). Nitrate was first reduced by nitrate reductase to nitrite and then nitrite was determined spectrophotometricaly by the Griess reaction [10]. Griess reagent, the mixture (1:1) of 0.2% naphthyetylene-diamine and 2% sulfonamide in 5% phosphoric acid, gives a red-violet diazo dye with nitrite and is measured at 550nm. The NO_3 + NO_2 values are given as µM.

Determination of CuZn SOD activity

CuZn SOD activity was determined according to the method of Sun et al. [11], based on the inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine–xanthine oxidase used as a superoxide generator. One unit of SOD was defined as the amount of protein that inhibits the rate of NBT reduction by 50%. SOD activity was expressed as ng/L.

Other analyses

Erythrocyte sedimentation rate (ESR) was measured according to Westergren method with an established normal range 0–20 mm/h. Serum levels of C-reactive protein (CRP) were measured by nephelometry (Behring Latex Enhanced on the Behring Nephelometer BN-100, Behring Diagnostic, Germany) with a sensitivity of 0.1 mg/L. Leukocyte levels in blood were obtained with automatic hematology analyzer (Beckman Coulter). Serological examinations were made to discover the presence of rheumatoid factor (RF) in immunoglobulins (IgM), using Waaler–Rose's test, with>8 as a positive level. Antinuclear Antibody (ANA) was measured by an indirect immunofluorescence technique, where samples and controls are incubated with the HEp-2 substrate. The unreacted antibodies are washed off and then an appropriate fluorescent labelled conjugate is applied. Unbound conjugate is washed off as before. Slides are viewed with a fluorescence microscope, and positive samples give rise to apple-green fluorescence which corresponds to areas of the HEp-2 cell where autoantibody has bound (HEp-2 ANA Kits, FK 001, FS 001).

Statistical analysis

All statistical analyses were performed using SPSS version 16.0 software (SPSS). Data are expressed as means ± SD. Groups were compared using Mann-Whitney U test and Student's t test for parametric data. For all statistical evaluation of the results, P values <0.05 were considered significant.

Results

Table 1 shows the analysed parameters and statistical significance in RA patients and healthy controls. As inflammatory markers; ESR and leukocyte count were significantly higher in RA patients with respect to controls. Both serum and

synovial fluid NO levels were significantly higher and CuZn SOD activity significantly lower in RA patients.

Table 2 shows the analyzed parameters and statistical significance when RA patients are classified according to DAS activity. In the moderate activity RA group (n=13), leukocytes and ESR and CRP levels were found to be significantly higher than low activity group (n=7). RA patients with DAS≥2.7 had significantly higher synovial fluid NO level and lower CuZn SOD activity than those with DAS<2.7. Synovial fluid NO level was significantly higher than serum NO level in moderate disease activity RA patients.

Table 1: Demographic and clinical data and statistical significance in RA patients and control group

	Control (n = 15)	RA (n = 20)
Age (years)	37.06 ± 9.73	40.20 ± 13.80
Sex (F/M)	8/7	13/7
RF, +,-	NA	11/9
ANA, +,-	NA	5/15
ESR (mm/h)	18.87 ± 5.21	49.00 ± 26.23 ***
Leukocytes (10^3/L)	7.14 ± 1.87	9.85 ± 2.51 **
CRP (mg/dL)	NA	36.64 ± 35.75
NO (μM)		
Serum	29.01 ± 4.58	38.64 ± 10.94 **
Synovial fluid	28.23 ± 13.89 [a]	38.79 ± 6.22 *
CuZn SOD (ng/L)	304.64 ± 66.22	232.74 ± 104.66 *

NA non assessed, NO nitric oxide, SOD superoxide dismutase, RF rheumatoid factor, ANA antinuclear antibody, ESR erythrocyte sedimentation rate, CRP C reactive protein,
a Control synovial fluid is obtained from non-arthritic group
** P<0.05 ** P<0.01 *** P<0.001*

Table 2: Demographic and clinical data and statistical significance in RA patients classified according to DAS activity

Intra group RA patients comparison		
	Low disease activity (DAS<2.7) (n = 7)	Moderate disease activity (DAS≥2.7) (n = 13)
Age (years	39.71 ± 11.13	40.46 ± 15.47
DAS	2.45 ± 0.09	4.41 ± 0.71***
RF, +,_	2/5	9/4
ANA, +,_	2/5	9/3
ESR (mm/h)	25.43 ± 12.99	56.31 ± 23.67***
Leukocytes (10^3/L)	8.17 ± 1.37	10.76 ± 2.55*
CRP (mg/dL)	23.30 ± 22.47	48.74 ± 37.36*
NO (μM)		
Serum	41.11 ± 6.79	37.30 ± 3.46 a*
Synovial fluid	34.19 ± 4.58	40.13 ± 2,94 *
CuZn SOD (ng/L)	298. 29 ± 91,67	197.45 ± 96.36 *

DAS disease activity score, ESR erythrocyte sedimentation rate, CRP C-reactive protein, RF Rheumatoid factor, ANA antinuclear antibody, Nitric oxide (NO), SOD (superoxide dismutase)
a: Synovial fluid NO level were compared with serum NO level in moderate disease activity RA patients.
**P<0.05, **P<0.01, *** P<0.001*

Table 3: Demographic and clinical data and statistical significance in RA patients classified according to RF, ±

Intra group RA patients comparison		
	RF (-) (n = 9)	RF (+) (n = 11)
Age (years)	37 ± 12.14	42.82 ± 15.07
ANA, +,-	2/7	3/11
ESR (mm/h)	47.67 ± 29.12	50.09 ± 25.03
Leukocytes (10^3/L)	8.83 ± 1.31	10.7 ± 1,9 *
CRP (mg/dL)	11.23 ± 6.43	50,02 ± 36,16 **
NO (μM)		
Serum	39.27 ± 11.39	38.11 ± 11.08
Synovial fluid	35.55 ± 5.12	40.09 ± 3.84 *
CuZn SOD (ng/L)	267.55 ± 61.25	205.16 ± 61.63 **

RF Rheumatoid factor, ESR erythrocyte sedimentation rate, CRP C-reactive protein, ANA antinuclear antibody, NO nitric oxide, SOD (superoxide dismutase),
** P<0.05 ** P<0.01*

Table 4: Demographic and clinical data and statistical significance in RA patients classified according to CRP levels

Intra group RA patients comparison		
	CRP≤ 3 mg/dL (n=6)	CRP> 3 mg/dL (n=14)
Age (years)	36 ± 11.56	42 ± 14.68
CRP (mg/dL)	5.74 ± 0.64	44.07 ± 33.61 **
RF, +,-	2/4	9/5
ANA, +,-	3/2	2/13
ESR (mm/h)	35 ± 16.88	54.29 ± 14.8 **
Leukocytes (10^3/L)	10.1 ± 2.54	9.75 ± 2.59
NO (μM)		
Serum	34.14 ± 5.08	40.56 ± 8.16 *
Synovial fluid	38.95 ± 4.18	37.67 ± 7.47
CuZn SOD (ng/L)	317.4 ± 50.83	196.46 ± 70.78 ***

CRP C-reactive protein, ESR erythrocyte sedimentation rate, RF Rheumatoid factor, ANA antinuclear antibody, NO nitric oxide, SOD superoxide dismutase
** P<0.05 ** P<0.01*

Comparison of RA patients according to the presence of RF revealed, significantly higher CRP and leukocyte levels in RF (+) patients (n=11). Synovial fluid NO level was significantly higher and SOD activity significantly lower in RF (+) patients compared to RF (-) ones (Table 3).

When RA patients with CRP>3 mg/dL were compared with those below 3mg/dL, serum NO level was significantly higher and SOD activity significantly lower in RA patients with CRP>3 mg/dL. Among inflammation markers; ESR was significantly higher in RA patients with CRP> 3 mg/dL (Table 4).

Grouping RA patients according to ESR revealed significantly high CRP and leukocyte count in the subgroup with ESR> 30 mm/h. In this group significantly lower serum NO level and SOD activity were observed compared to RA patients with ESR ≤ 30mm/h (Table 5).

Table 5: Demographic and clinical data and statistical significance in RA patients classified according to ESR levels

Intra group RA patients comparison		
	ESR ≤ 30 mm/h (n=6)	ESR > 30 mm/h (n=14)
Age (years)	43.83 ± 11.09	36.93 ± 14.76
ESR (mm/h)	18.5 ± 7.72	62.07 ± 19.23 **
RF, ±	3/3	8/6
ANA, ±	3/3	3/11
Leukocytes (10^3/L)	8.52 ± 1.14	10.43 ± 1.72 *
CRP (mg/dL)	16.05 ± 18.13	39.65 ± 27.83 *
NO (μM)		
Serum	46.77 ± 14.2	35.15 ± 7.33 *
Synovial fluid	37.18 ± 2.70	38.42 ± 7.73
CuZn SOD (ng/L)	278.4 ± 40.61	213.17 ± 65.64 *

ESR erythrocyte sedimentation rate, CRP C-reactive protein, RF Rheumatoid factor, ANA antinuclear antibody, NO nitric oxide, SOD (superoxide dismutase)
** P<0.05 *** P<0.001*

Discussion

This study demonstrates significantly increased serum and synovial fluid NO levels and decreased erythrocyte CuZn SOD activity in RA patients compared to healthy controls. As to inflammatory response; ESR and leukocyte number were significantly higher in RA patients. Classification of RA patients according to DAS activity revealed significantly higher synovial fluid NO level and significantly lower CuZn SOD activity in moderate disease activity group (DAS>2.7). Furthermore we failed to find any significant correlation among inflammatory markers, DAS activity and NO concentration.

Both the pro-inflammatory and potent anti-inflammatory / antioxidative properties of NO, as a bifunctional modulator, led to NO paradox in discussions about inflammatory processes.

Several lines of evidence indicate the importance of RNS in the pathogenesis of tissue damage in RA. The proinflammatory effects of NO include augmentation of vascular permeability in inflamed tissues, generation of destructive free radicals such as peroxynitrite and hydroxyl radical, induction of cyclooxygenase and inflammatory cytokines like TNF-α and IL-1 and chondrocyte apoptosis [12,13].

NO has potent anti-inflammatory effects by down regulating the activity of neutrophils and macrophages, preventing neutrophil adhesion and modulating T-cell function .NO is a key mechanism that limits oxidative injury to mammalian cells by inhibiting lipid peroxidation effectively. Inhibition of cytocrome oxidase in mitochondria by NO favours superoxide anion formation. Thus the release of proinflammatory cytokines, together with ROS and NO from the sites of inflammation, is associated with increased oxidative stress, consistent with inhibition of antioxidant enzymes, most notably decreased CuZn SOD expression [14-17].

In line with our finding; Choi JW [18] reported increased NO production in RA patients, without any correlation with laboratory parameters of disease activity and Marklund SL [19] observed very low SOD concentration in joint fluid. G Nagy et al [20] also observed no correlation between clinical disease activity and NO production in RA patients.

However Onur Ö et al [21] found elevated levels of nitrate in rheumatoid arthritis, with a significant correlation between serum nitrate concentrations and number of tender joints, number of swollen joints, Ritchie articular index, DAS score and CRP level. Similar to above findings; Ueki et al [22] demonstrated a significant relationship between serum NO levels and disease activity in rheumatoid arthritis. The parameters of disease activity that correlated with serum NO significantly were morning stiffness, number of tender swollen joints and CRP levels, whereas ESR and the Landsbury index revealed no significant relationship.

In contrast to our findings, Sarban S et al [23] reported that plasma and synovial fluid NO levels were similar in RA patients and healthy controls.

Increased concentrations of nitrite in synovial fluid and serum of RA patients were observed by Farrell AJ et al. [24] It was suggested that significantly higher synovial fluid nitrite than serum nitrite implied NO synthesis by the synovium. In our study in RA patients serum and synovial fluid NO levels did not differ significantly. However, our findings of significantly higher synovial fluid NO than serum NO concentration in moderate disease activity RA patients support those of Farell AJ et al. [24] localization studies have shown up-regulation of; NOS expression in synovial lining cells, chondrocytes and blood vessels in joint tissues obtained from RA patients [25].

As to inflammatory response both leukocyte, CRP and ESR levels were synificantly higher in DAS≥2,7

RA group. When RA patients were grouped as CRP<3 mg/dL vs CRP>3 mg/dL, serum NO level was significantly higher and CuZn SOD activity lower in CRP>3 mg/dL group. CRP level was found to be significantly higher in CRP>3 mg/dL group.However, Choi JW [18] observed no significant difference in NO level when RA patients were grouped as CRP<0.7 vs CRP>3.0, but significantly higher CRP, ESR and RF values in CRP≥3.0 mg/dL group. Elevated CRP levels in RA patients were reported to be associated with increases in cell adhesion molecules related with endothelial dysfunction.

Our findings of significantly high CRP levels in RA patients with ESR >30mm/hr in comparison to those <30 mm/hr are in line with the observations of J.W.Choi [18] who reported high CRP and RF levels in RA patients with ESR >40 mm/hr with respect to those lessthan 15.0 mm/hr.

Our findings reveal increased serum and synovial fluid NO levels and decreased CuZn SOD activity in RA patients. Induced NO, in addition to being a final common mediator of inflammation, is essential for the up-regulation of the inflammatory response, as observed by significantly high ESR and leukocyte levels.

The authors declare no conflict of interest.

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