Turkish Journal of Medical Sciences

Volume 40 | Number 2 Article 5

1-1-2010

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TÜBİTAK

Original Article

Turk J Med Sci
2010; 40 (2): 191-197
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E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-0809-32

Serum nitric oxide and peroxynitrite levels in adult sero-positive rheumatoid arthritis treated with disease modifying antirheumatic drugs: a preliminary report

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Aim: The contribution of inducible nitric oxide synthase (iNOS) to oxidative/nitrative stress is well documented in inflamed joints. Nitric oxide stimulates the synthesis of proinflammatory mediators and cytotoxic molecules with a pivotal role in apoptosis at the joint of rheumatoid arthritis. This study aimed to assess the serum levels of nitric oxide and peroxynitrite in sero positive rheumatoid patients treated with disease modifying antirheumatic drugs.

Materials and methods: Sixteen known patients with sero-positive rheumatoid arthritis fulfilling the criteria of the American College of Rheumatology and on disease modifying antirheumatic drugs were allocated from the consultant clinic of rheumatology at Al-Yarmouk Teaching hospital in Baghdad, Iraq, from October 2004 to May 2005. The serum levels of nitric oxide and peroxynitrite were determined for patients as well as for another 16 healthy individuals serving as controls.

Results: The mean serum levels of nitric oxide (116.9 μmol) and peroxynitrite (7.3 μmol) were significantly higher than the controls' levels of 46 μmol and 2.5 μmol, respectively. Females had non-significantly lower serum nitric oxide and higher serum peroxynitrite than corresponding males. Patients older than 50 years had non-significantly higher serum nitric oxide and lower serum peroxynitrite levels than those younger than 50 years old. There was a non-significant correlation between the serum levels of each of nitrogen species and the duration of disease or erythrocyte sedimentation rate as a marker of disease activity.

Conclusion: Sero-positive rheumatoid patients treated with disease modifying antirheumatic drugs have significantly high serum nitric oxide and peroxynitrite levels that are not related to the duration or disease activity.

Key words: Rheumatoid arthritis, nitric oxide, peroxynitrite

Introduction

Nitric oxide (NO) is one of the few gaseous signaling molecules known (1). It is involved in many physiological and pathological processes within the body, both beneficial and detrimental (2,3). Appropriate levels of NO production are important in protecting organs from ischemic damage (4), whereas chronic expression of NO is associated with various malignancies and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (5,6). Genetic factors including endothelial nitric oxide synthase (eNOS) were implicated in pathogenesis of rheumatoid arthritis, and extra-articular manifestations of rheumatoid arthritis were significantly greater among the carriers (7).

Received: 17.09.2008 – Accepted: 20.08.2009

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There is growing evidence that nitrative injury plays an important role in the pathogenesis of rheumatoid arthritis. In the experimental model of rheumatoid arthritis such as rat adjuvant and collagen induced arthritis, NO production was significantly elevated (8), and the plasma levels of 3-nitrotyrosine were significantly correlated with the severity of the inflammatory response (9). Moreover, in the experimental model, small doses of NO donors attenuate joint inflammation and hyperalgesia while higher doses produce infiltration of mononuclear cells and cartilage erosion (10). Raised levels of reactive nitrogen species in serum and synovial fluid have been reported in patients with rheumatoid arthritis (11,12).

The correlation between reactive nitrogen species levels and disease activity is still not confirmed. In juvenile rheumatoid arthritis, the significant high serum NO level is correlated with clinical indices of inflammation and laboratory parameters of disease activity (12,13), while such a correlation is not definite in adults (14-16).

In synovial fluid, the NO production is stimulated by immune complex especially in acute rheumatoid arthritis and the increased intraarticular NO reflects abnormalities in the immune system regulation in the joint (14). The intraarticular granulocytes express inducible nitric oxide synthase [iNOS], and thus contribute to the intraarticular production of NO (17).

The assessment of nitrosative stress in rheumatoid arthritis was studied by measuring the related markers. These include serum and synovial fluid nitrate (an indirect measure of nitric oxide production) (11), urinary nitrate:creatinine ratio (an assessment of endogenous nitric oxide production) (18), serum N^G-hydroxy-L-arginine (an index of increased nitric oxide synthase activity) (19), and 3-nitrotyrosine level in serum or synovial fluid (specific marker of nitrosative damage) (20-22). Previous studies did not assess the serum peroxynitrite level in rheumatoid patients.

This study aimed to assess the serum levels of NO and peroxynitrite in patients with rheumatoid arthritis treated with disease modifying antirheumatic drugs (DMARDs).

Materials and methods

This study was conducted at the Department of Pharmacology in cooperation with Al-Yarmouk Teaching Hospital and the Department of Biochemistry, College of Medicine, Al-Mustansiriyia University in Baghdad, Iraq, from October 2004 to May 2005. After obtaining permission from the local ethics committee and informed patient consent, patients that fulfilled the criteria of the American College of Rheumatology (formerly The American Rheumatism Association) (23), from the consultant outpatient clinic at Al-Yarmouk Teaching Hospital, were enrolled in the study. The criteria of inclusion included sero-positive adult rheumatoid arthritis, those treated with DMARDs [methotrexate® tablet 7.5 mg/week, chloroquine sulfate (Nivaquine)® tablet 200 mg/day, cyclosporine (Neoral)® oral solution 2 mg/day, penicillamine (Distamine)® 250 mg /day] either individually or in combination, and the patients were allowed freely to take nonsteroidal anti-inflammatory drugs when needed. The criteria of exclusion were concomitant diseases including hypertension and diabetes mellitus. A consultant rheumatologist assessed each patient clinically, radiologically, and serologically.

Sixteen patients (12 females and 4 males) with a median age of 50 years were included in the study. The median duration of the disease was 5 years. The distribution of patients in respect of taking DMARDs was as follows: methotrexate 5, chloroquine sulfate 1, penicillamine 1, cyclosporine A 1, and a combination of methotrexate and chloroquine sulfate 8. The erythrocyte sedimentation rate at the time of the study was taken as a routine marker of disease activity. The mean ± SD of erythrocyte sedimentation rate was 59.6 ± 30.8 mm/h. In addition, 16 subjects matched to the patient group with regard to age and gender were allocated randomly from the staff departments at the college of medicine. All subjects were examined and investigated by routine laboratory tests and they appeared healthy.

Venous blood samples were collected from patients and healthy subjects. The sera were separated by centrifugation (3000 rpm for 3 min) and immediately processed for NO and peroxynitrite assay. NO in the serum was determined as described by Navarro-Gonzalvez et al. (24) by measuring the concentration of

nitrates. The reduction of nitrate to nitrite by cadmium is the basis for this method. The Griess reaction was used to determine the nitrite, which reacts with a Griess reagent to form Griess chromophore. In brief, 150 µL of serum was deproteinized by adding 250 µL of 75 mM $ZnSO_4$ solution, stirring and centrifuging at 10,000 ×g for 5 min at 25 °C. Then we added 350 µL of 55 mM NaOH and stirred and centrifuged the solution at 10,000 ×g for 5 min at 25 °C. We recovered the supernatant, which was free from turbidity, and diluted 750 µL of this with 250 µL of glycine buffer (45g/L, pH 9.7). We then added 2-2.5 g of freshly activated cadmium granules to 1 mL of pretreated deproteinized serum and stirred this continuously for 10 min. After that, we transferred 200 µL of the treated serum into another tube and added Griess reagent (750 µL of 25 mg N-naphthylenediamine in 250 mL of distilled water and 800 µL of 5 g of sulfanilic acid in 500 mL of 3 M HCl). The absorbance of the sample was recorded at 340 nm by SpeCol® spectrophotometer. The concentration of serum NO (µmol) was calculated in respect to the standard lithium nitrate absorbance concentration curve with a best fit line equation of: Absorbance (O.D.) = 0.0018 + 0.0002 × concentration (r = 0.997).

Serum peroxynitrite level was determined according to the method described by Beckman et al. (25), cited by VanUffelen et al. (26). Peroxynitrite mediated nitration of phenol, resulting in nitrophenol formation, formed the basis of the peroxynitrite assay. In brief, 10 µL of serum was placed in a glass test tube and added to 5 mM phenol in 50 mM sodium phosphate buffer to a final volume of 2 mL and mixed well. This was then incubated for 2 h at 37 °C, followed by the addition 15 µL of 0.1 NaOH and mixed. We then recorded the absorbance of the sample at 412 nm by SpeCol® spectrophotometer [PGH, Radi Fernesehen Electro, Germany]. We calculated the yield of nitrophenol from $\varepsilon = 4400\ M^{-1}\ cm^{-1}$.

All the chemicals used in this work were of Analar grade, dissolved in distilled water, and prepared freshly at the time of assay.

The results are expressed as mean, median, standard deviation [SD] and 95 % confidence interval**.** Student's unpaired 2-tailed "t" test and a simple correlation test with linear least squares were used. $P \leq 0.05$ was considered statistically significant.

Results

Table 1 shows the demographic characteristics of the subjects. There was no significant difference between the control and rheumatoid patients regarding gender, age, body mass index, or smoking habit.

The sero-positive rheumatoid patients treated with DMARDs had a significantly high serum level of NO that was more than 2-fold that of the controls and their serum peroxynitrite level was approximated 3 times that of the controls (Table 2).

Males with rheumatoid arthritis tended to have nonsignificantly (95% C.I. 7.85-4, P > 0.05) high serum levels of nitric oxide as compared with females (160.35 ± 21.746 µmol vs. 95.918 ± 48.116 µmol respectively) while the picture is reversed with peroxynitrite i.e. females had nonsignificantly (95% C.I. 7.85-40.15, P > 0.05) higher values than corresponding males [7.821 ± 4.857 µmol vs. 5.511 ± 4.184 µmol]. Patients older than 50 years had nonsignificantly (95% C.I. 9.43-45.57, P > 0.05) higher levels of NO (135.1 ± 31.503 µmol) and

Table 1. The characteristics of the subjects.

	Controls (n = 16)	Rheumatoid patients treated with DMARDs (n = 16)
Gender		
Male	4	4
Female	12	12
Age (year)		
<50	8	9
>50	8	7
Weight (kg)	70.3 ± 10.2	72.6 ± 14.8
Height (m^2)	1.61 ± 0.15	1.62 ± 0.12
Body mass index (kg/m^2)	27.1 ± 5.9	27.7 ± 6.6
Smoking		
Active	4	3
Passive	10	7
Alcohol consumption	0	0
Duration of illness (year)		
Range	–	2-15
Median	–	5

nonsignificantly (95% C.I. 9.43-45.57, P > 0.05) lower peroxynitrite levels (6.454 ± 3.43 μmol) than those under 50 years old (108.645 ± 55.292 μmol and 7.603 ± 5.258 μmol respectively). There was no significant negative correlation between the serum NO and the duration of the disease (r = -0.0192, a = 118.39, b = -0.229) or erythrocyte sedimentation rate (r = -0.0259, a = 141.88, b = -0.0419) (Figure 1). Moreover, peroxynitrite did not significantly correlate with the duration of the disease (r = -0.172, a = 8.494, b = -0.1942) or the erythrocyte sedimentation rate (r = 0.321, a = 4.329, b = 0.04189) (Figure 2).

Table 2. Serum levels of NO and peroxynitrite.

Nitrogen species	Controls (n = 16)			Rheumatoid patients treated with DMARDs (n = 16)		
	Mean	Median	SD	Mean	Median	SD
Nitric oxide (μmol)	46	48.5	15.75	116.91	131.6*	49.63
Peroxynitrite (μmol)	2.542	2.727	0.599	7.348	7.386**	4.676

* P < 0.001 (t value = 6.575)
** P < 0.001 (t value = 4.110)

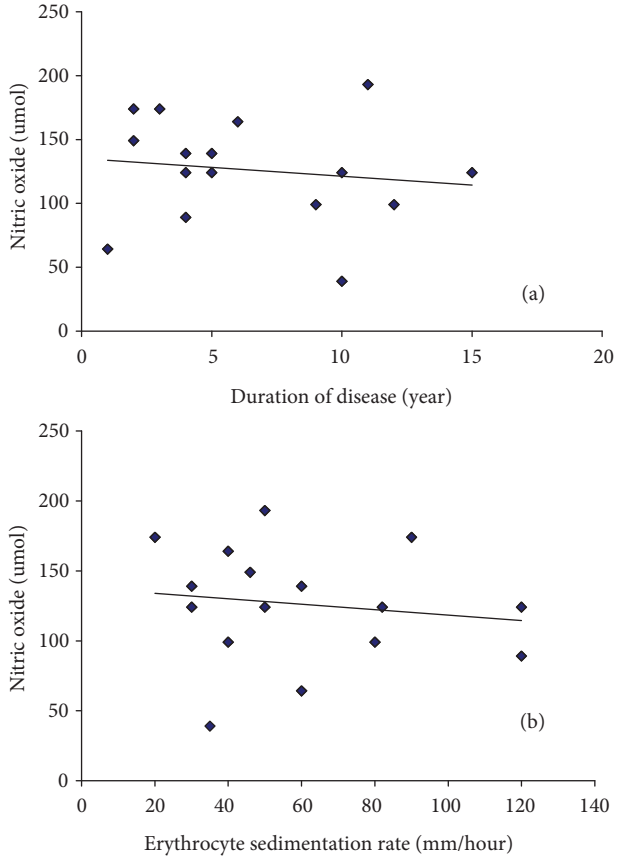

Figure 1. Nonsignificant correlation between duration of disease [A] or erythrocyte sedimentation rate [B] with serum nitric oxide in sero-positive rheumatoid arthritis patients.

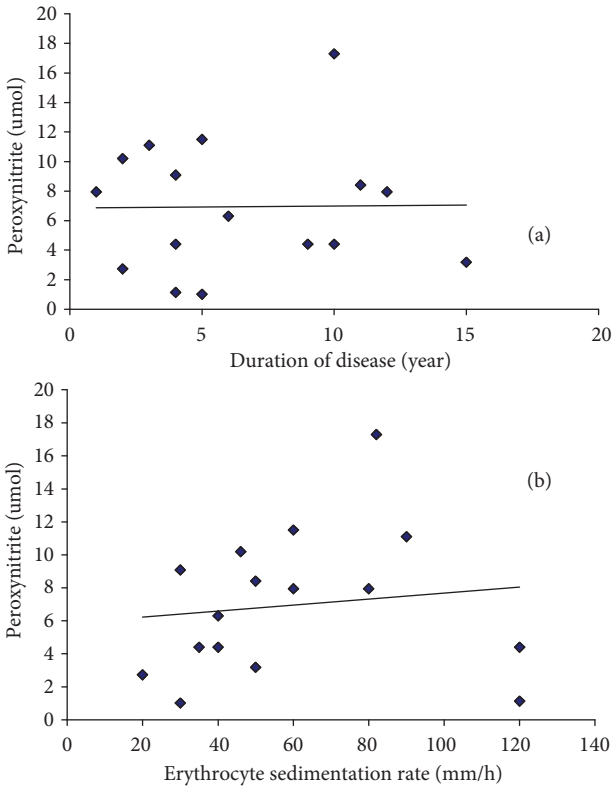

Figure 2. Nonsignificant correlation between duration of disease [A] or erythrocyte sedimentation rate [B] with serum peroxynitrite in sero-positive rheumatoid arthritis patients.

Discussion

The results of this work show overproduction of nitrogen species in sero-positive rheumatoid patients treated with DMARDs, reflected by significantly high serum NO and peroxynitrite levels. Under normal conditions iNOS activity is very low, but it is stimulated during inflammation by cytokines such as tumor necrosis factor (TNF-α). Functional iNOS gene polymorphisms have been associated with susceptibility to rheumatoid arthritis (6). This explains the high level of NO in this study. Moreover, the presence of peroxynitrite, a redox derivative of NO, enhances the inflammatory response by sustaining the nuclear localization of nuclear factor-kappaB($NF_{κB}$) (27).

It is well known that the expression of inducible NO synthase enzyme, in arthritis, is upregulated by many proinflammatory mediators like infernos and interleukins (28). Moreover, in rheumatoid patients HLA-DRB1 alleles tended to trigger nitric oxide mediated signaling events (29). The generation of neo-antigenic determinants by reactive oxygen and nitrogen species may contribute to epitope spreading in autoimmunity (30). The oxidation of amino acid by peroxynitrite increases the antigenicity of immunoglobulin-G, generating ligands for which autoantibodies show higher activity (30). This could explain the high levels of nitrogen species in sero-positive rheumatoid arthritis but it does not explain the high levels in patients treated with DMARDs. Matyska-Piekarska et al. (2006) found that the favorable effect of DMARDs on articular damage is related to their effects on reactive oxygen species (31). Therefore, the results of this work show that DMARDs seem to have no effect on reactive nitrogen species. Recently, Gonzalez-Gay et al. (6) reported that infliximab, a TNF-α blocker, significantly reduced the serum levels of nitrogen species and those who do not response to anti-TNF- α therapy could experience arthritis that is perpetuated by the B-cell more than the T-cell and highlighted that nitrogen species are not the only factor involved in the pathogenesis of rheumatoid arthritis (32).

Several authors assessed nitrosative stress in rheumatoid patients in sera in terms of finding significant high levels of NO (14,33-35), nitrate (15,18), nitrite (11,16,33), and nitrotyrosin (20) or nonsignificant changes in plasma nitrite level (36), but there is no report about serum peroxynitrite as with this study. The significant high serum peroxynitrite, the product of the reaction of nitric oxide and superoxide anion, can serve as an indicator for simultaneous assessment of reactive oxygen and nitrogen species.

In respect to the correlation between serum nitrogen species levels and markers of disease activity, the present results are in accordance with others (16,28). Furthermore, polymorphisms in genes coding for catalase 262CC and TNF-α 308GG but not iNOS (NOS2A) alone or in combination influence the activity of rheumatoid arthritis (37), i.e. no relation between disease activity and nitrogen species as demonstrated in this study. Therefore, it is possible to postulate that nitrogen species are essential for the upregulation of the inflammatory response (38) but do not serve as a prognostic marker. It is concludeed that rheumatoid patients treated with DMARDs have significantly high serum nitrogen species that are unrelated to the duration or the activity of disease. Further research is recommended to elucidate the difference in the magnitude of nitrogen species in rheumatoid patients between those that are sero-positive and sero-negative.

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