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Oxidation of Uric Acid in Rheumatoid Arthritis: Is Allantoin a Marker of Oxidative Stress?

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Free radicals are implicated in many diseases including atherosclerosis, cancer and also in rheumatoid arthritis. Reaction of uric acid with free radicals, such as hydroxyl radical and hypochlorous acid (HOCl) results in allantoin production. In this study, we measured the serum allantoin levels, oxidation products of uric acid, as a marker of free radical generation in rheumatoid arthritis. Fasting blood samples were obtained from 21 rheumatoid patients and 15 healthy controls. In this study, the serum allantoin and uric acid levels were measured by a gas chromatography–mass spectrometry method and the ratios were calculated. The mean allantoin and uric acid levels and ratios in the patient group were 22.1 ± 11.3, 280.5 ± 65.0 and $8.0 \pm 3.7\,\mu M$, while in the control group they were 13.6 ± 6.3, 278.3 ± 53.6 and $4.9 \pm 2.1\,\mu M$, respectively. The effects of gender, age, menopausal status, duration of disease and medications on serum allantoin and uric acid levels of the patient and control groups were studied. Our results suggest that uric acid acts as a free radical scavenger and thus is converted to allantoin. Increased allantoin levels suggest the possible involvement of free radicals in rheumatoid arthritis.

Keywords: Uric acid; Allantoin; Hypochlorous acid; Rheumatoid arthritis

INTRODUCTION

During the last 2–3 decades, several studies have focused on oxidative stress in rheumatoid arthritis.[1–12] The characteristic feature of rheumatoid arthritis is persistent inflammation. Different pathways can cause increased formation of reactive oxygen species at the site of inflammation, including superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) and hypochlorous acid (HOCl).[1,4,13]

The nature of free radicals made direct measurement difficult, and has resulted in the estimation of free radical activity by its effects on biological systems, e.g. by measuring the products of free radical attack on macromolecules and lipids or their effect on the antioxidant defense system. Several analytical methods have revealed the presence of allantoin in serum or plasma, as the first and major product of uric acid oxidation.[12,14–16]

Allantoin is the catabolic end product of purines in some mammals. It is formed by the action of the enzyme uricase on uric acid. In humans, no enzyme which oxidizes uric acid is known, however, uric acid may be transformed into allantoin by chemical oxidation or oxygen radicals.[17–19]

Serum or plasma allantoin levels in several studies have previously been reported as a marker of free radical activity.[12,14,20,21] The major aim of this study was to investigate the possible involvement of free radicals in rheumatoid arthritis by measuring allantoin levels as a marker of uric acid oxidation. The secondary aim was to investigate the effects of several parameters on uric acid and allantoin levels, including gender, age, menopausal status, duration of disease and medications.

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MATERIALS AND METHODS

Study Population

Fifteen healthy subjects (12 female, 3 male; mean age ± SD = 55.3 ± 10.3) were included in this study. Twenty one rheumatoid arthritis patients (17 female, 4 male; mean age ± SD = 54.7 ± 14.0) admitted to the Physical Medicine and Rehabilitation Department of Gazi University Hospital in Ankara were studied. Patients were classified into three groups according to the duration of disease: Group 1, newly-diagnosed patients ($n = 3$); Group 2, patients suffering from rheumatoid arthritis for 0–5 years ($n = 7$); Group 3, patients suffering from rheumatoid arthritis for more than 5 years ($n = 11$). Patients in Groups 2 and 3 had well-controlled, although active, rheumatoid arthritis. Sixteen of the 21 patients were treated either only with methotrexate or methotrexate plus salozopyrin and steroidal medicines or only salozopyrin and steroidal medicines. The other patients had not been treated. Informed consent was obtained from all subjects before the study. The study was approved by the Gazi University Hospital Ethics Committee. All patients were diagnosed as having rheumatoid arthritis according to the American Rheumatism Association (ARA) criteria.[22]

Sample Collection and Storage

Blood samples were collected from patients and controls following an overnight's fast, and then centrifuged as soon as possible at 2000*g* for 10 min. Serum samples were stored in the dark at −70°C until the analysis.

Chemicals

Allantoin, uric acid, hydrochloric acid and *N*-(*tert*-butyl-dimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were obtained from Merck (Darmstadt, Germany). Dimethylformamide (DMF) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile was obtained from LabScan (Dublin, Ireland). SPE Cartridges (100 mg, 1 ml) were acquired from Alltech (Illinois, USA).

GC-MS Instrumentation and Conditions

HP 6890 Gas Chromatograph and HP 5972A mass spectrometer (Hewlett Packard, Germany) were used in the analysis. The samples were applied onto HP-5MS capillary column (25 m × 0.25 mm i.d., 0.33 μm film thickness) (Hewlett Packard) with helium as carrier gas (flow rate 1.2 ml/min). Injection port temperature was 280°C and initial oven temperature was 150°C rising to 270°C at 20°C/min for 6 min. The ions at m/z 398 and 400 for allantoin and m/z 567 and 569 for uric acid were monitored under selective ion recording conditions (ionization energy, 70 eV) with a 30 ms dwell time on each ion.

Sample Preparation

The levels of allantoin and uric acid in serum were determined by gas chromatography–mass spectrometry (GC-MS) according to the method of Chen *et al.*[23] with small modifications. The serum samples (250 μl) was mixed with 250 μl of acetonitrile and centrifuged at 10,000*g* for 10 min. Extraction column was conditioned with water, then 400 μl supernatant with internal standard was applied to the column. Allantoin and uric acid were eluted with 1 ml 0.1 M HCl. A 500-μl volume of the eluate was transferred to a vial and dried at 90°C under nitrogen. The *tert*-butyldimethylsilyl (TBDMS) derivatives of allantoin and uric acid were formed by reacting with 80 μl of DMF–MTBSTFA (1:1) at 130°C for 20 min. One microliter of solution was injected onto a GC-MS column.

Statistical Analysis

Data were expressed as the mean ± SD, and statistical analyses were performed by Student's *t*-test and Mann–Whitney U test (for groups of small numbers). Pearson correlation coefficients were calculated for the relationship between allantoin and uric acid. Statistical analyses were performed with a SPSS 10.0 Package (SPSS Inc., USA).

RESULTS

Table I presents baseline characteristics and laboratory values of the patients and controls. Table II shows the mean allantoin and uric acid levels and allantoin/uric acid ratio in the patients and controls. The serum allantoin levels and ratio values of patients were significantly higher than those of controls ($p < 0.01$ and 0.005, respectively). However, uric acid levels were unchanged in both groups ($p > 0.05$). There is a positive, but not statistically significant correlation between allantoin and uric acid levels in the patients ($r = 0.39$, $p = 0.08$). When we analyzed the effects of gender on our parameters, we found higher allantoin levels in patient and control females than those in males ($p < 0.005$ and $= 0.06$, respectively) (Table III). Uric acid levels were similar in both groups. There was a positive correlation between age and uric acid in patients ($r = 0.846$, $p < 0.0001$) (Fig. 1). The younger people had lower uric acid levels in both patients ($p < 0.0001$) and controls ($p < 0.05$) (Table III). Serum allantoin levels did not show any significant change with age. Menopausal status also affected

TABLE I Baseline characteristics and laboratory values of the patients and controls

Variables	Patients ($n = 21$)	Controls ($n = 15$)	p-Value
Mean age (years)	54.7 ± 14.0	55.3 ± 10.3	NS
Female included (%)	81.0	80.0	–
Menopausal status (pre/post)	8/9	6/6	–
c-Reactive protein (mg/dl)	38.0 ± 9.9	–	–
Sedimentation (mm/h)	124.1 ± 40.2	–	–
Hemoglobin (g/dl)	12.5 ± 1.0	13.7 ± 0.5	0.0001
Total protein (g/dl)	7.2 ± 0.4	7.1 ± 0.2	NS
Albumin (g/dl)	4.3 ± 0.3	4.4 ± 0.3	NS
Creatinine (mg/dl)	0.9 ± 0.2	0.8 ± 0.1	NS

serum uric acid levels. Post-menopausal female had higher levels of uric acid in both patient and control groups ($p < 0.0001$ and 0.05, respectively) (Table III). In patients, serum allantoin levels were lower in pre-menopausal than in post-menopausal female, but not statistically significant. In addition, there was a positive correlation between allantoin and uric acid levels in pre-menopausal control female ($r = 0.894$, $p = 0.016$).

There is a positive correlation between duration of disease and allantoin levels ($r = 0.653$, $p = 0.001$) (Fig. 2). When we investigated allantoin levels in groups, Group 3 had higher levels than those in Groups 1 and 2, but not significantly so (Table III).

Serum concentrations of allantoin and uric acid did not show any significant changes with medications (Table III).

DISCUSSION

Rheumatoid arthritis is a chronic multisystem disease of unknown etiology. The characteristic feature of this disease is persistent inflammation. Neutrophils have the ability to generate a number of potentially toxic free radicals via the combined activities of NADPH oxidase and myeloperoxidase (MPO).[13,24,25] Some other cell types found within diseased joints may also possess and secrete some of these damaging products. $O_2^{\cdot-}$ is released at the site of the inflammation. It can react directly with several substrates or it can be dismutated to form hydrogen peroxide (H_2O_2). This compound is a substrate for MPO, which forms a potent oxidant, HOCl. Also, formations of other reactive species, including $HO^{\cdot}$, singlet oxygen ($^1O_2$), have been demonstrated.[4,18,24,26–28]

Uric acid is known as the main end product of purine nucleotide metabolism in humans. While uric acid is converted into allantoin by action of uricase in most mammals, there is no enzyme in humans to oxidize it further. Therefore, at first, it was thought to be only a waste product. After filtration by the kidney, however, the majority of uric acid is reabsorbed into plasma, indicating that it has a physiological role. Uric acid is one of the important intra- and extra-cellular antioxidants because it is found in plasma in higher concentrations than other antioxidants.[12,17,29–32] The antioxidant properties of uric acid have been attributed to its ability to react with potent biological oxidants to produce relatively stable products. It has been shown that HOCl and $HO^{\cdot}$ can react rapidly with uric acid to form allantoin. It is the most abundant and most stable oxidation product of uric acid, and may be a sensitive marker of oxidative stress *in vivo*.[18,19,30,33–35]

In several studies, the relationship between uric acid and rheumatoid arthritis was investigated. Bosmansky and Trnavsky found hyperuricemia in rheumatoid arthritis.[36] Hagfors *et al.* showed that uric acid was inversely related to indices of disease activity.[37] Hasegawe and Kuroda detected allantoin in rheumatoid patients, but not in controls.[38] In a small group, Grootveld and Halliwell showed higher allantoin levels and allantoin/urate ratio in rheumatoid patients than those in controls. They also observed unchanged uric acid levels in both groups.[12] In the present study, we also found similar results. In fact, the significant elevation of the serum allantoin levels and allantoin/urate ratio in the rheumatoid patients can indicate increased oxidation of uric acid.

Normally, it is known that serum uric acid levels are higher in male than in female.[39–41] However, serum uric acid levels in female increase at the time of or after menopause. It is suggested that estrogen may enhance excretion of uric acid via urine.[42] In our study, we could not find any difference between male and female for uric acid levels in patients and controls. In our opinion, this may result from a big difference of uric acid levels between pre- and post-menopausal female.

TABLE II The levels of allantoin and uric acid in patients with rheumatoid arthritis and controls

	Mean levels ± SD (95% confidence interval)		
	Allantoin (μmol/l)	Uric acid (μmol/l)	Allantoin/uric acid (%)
Patients ($n = 21$)	22.1 ± 11.3* (17.0–27.2)	280.5 ± 65.0 (250.9–310.0)	8.0 ± 3.7** (6.3–9.7)
Controls ($n = 15$)	13.6 ± 6.3 (10.1–17.0)	278.3 ± 53.6 (248.6–308.0)	4.9 ± 2.1 (3.8–6.0)

*$p < 0.01$, **$p < 0.005$.

TABLE III Mean levels (±SE) of serum allantoin and uric acid according to basic characteristics in patient and control groups

	Allantoin (μmol/l)				Uric acid (μmol/l)	
	n	Patients	*n*	Controls	Patients	Controls
Gender						
Female	17	24.0 ± 11.8	12	15.0 ± 6.0	281.2 ± 69.0	279.1 ± 54.1
Male	4	14.1 ± 1.5	3	7.6 ± 3.9	277.3 ± 52.6	275.2 ± 63.3
Age						
X ≤ 50	9	18.9 ± 9.9	5	15.3 ± 7.3	221.9 ± 27.0	247.4 ± 59.3
X > 50	12	24.6 ± 12.0	10	12.7 ± 5.9	324.3 ± 47.4	293.7 ± 45.9
Menopausal status						
Pre-menopause	8	19.5 ± 10.4	6	14.9 ± 6.6	224.0 ± 28.1	244.0 ± 53.6
Post-menopause	9	27.9 ± 12.1	6	15.2 ± 5.9	332.1 ± 51.3	314.1 ± 24.6
Duration of disease						
Group 1	3	15.6 ± 8.7		–	229.7 ± 38.7	–
Group 2	7	18.7 ± 10.2		–	291.0 ± 76.1	–
Group 3	11	26.0 ± 11.8		–	287.6 ± 61.4	–
Treatment status						
No	5	16.3 ± 8.0		–	284.5 ± 67.1	–
Yes	16	23.9 ± 11.7		–	279.2 ± 66.5	–

On the other hand, there are few published studies to show effects of gender on allantoin levels. Benzie *et al.* found similar allantoin levels in male and female in both NIDDM patients and controls.[14] Pavitt *et al.* found significantly higher allantoin levels in male as compared with female in a group of healthy subjects.[16] In our two unpublished studies (in review), we were unable to obtain consistent results about the relationship between gender and allantoin levels. In those studies, the mean allantoin levels in the cardiovascular patients were higher in male, while in lung cancer patients, allantoin levels were higher in female. In the current study, we found higher allantoin levels in male, but we could not comment on this result because of the small number of male.

We found lower uric acid levels in the younger people in both the patients and controls. Also, there was a positive correlation between age and uric acid levels in patients. Gathof *et al.* found lower levels of uric acid in younger female, but not so in male.[39] Our results are consistent with their findings. We could not find any study to support this relation in rheumatoid patients.

We found positive correlation between duration of disease and allantoin levels. Especially, we obtained higher levels of allantoin in Group 3 than in other

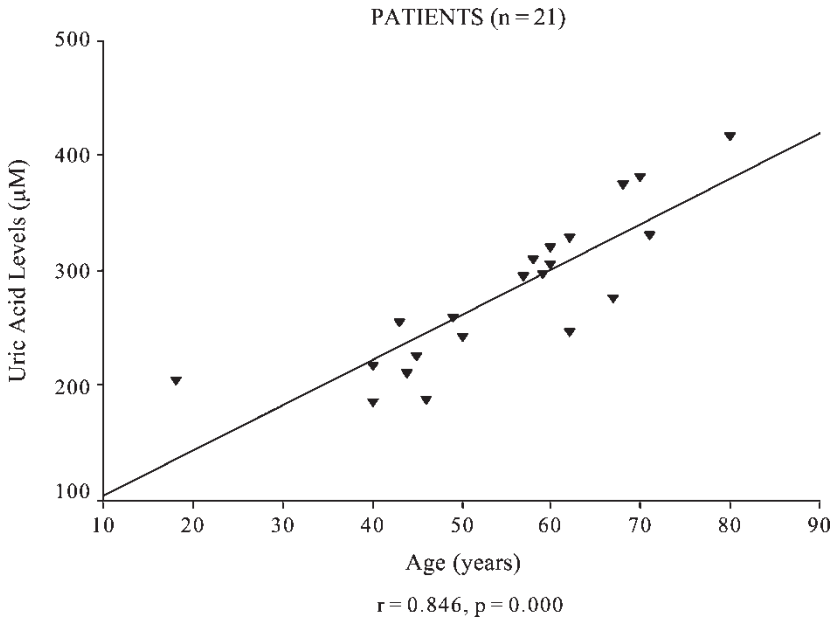

FIGURE 1 Correlations between uric acid and age in patients.

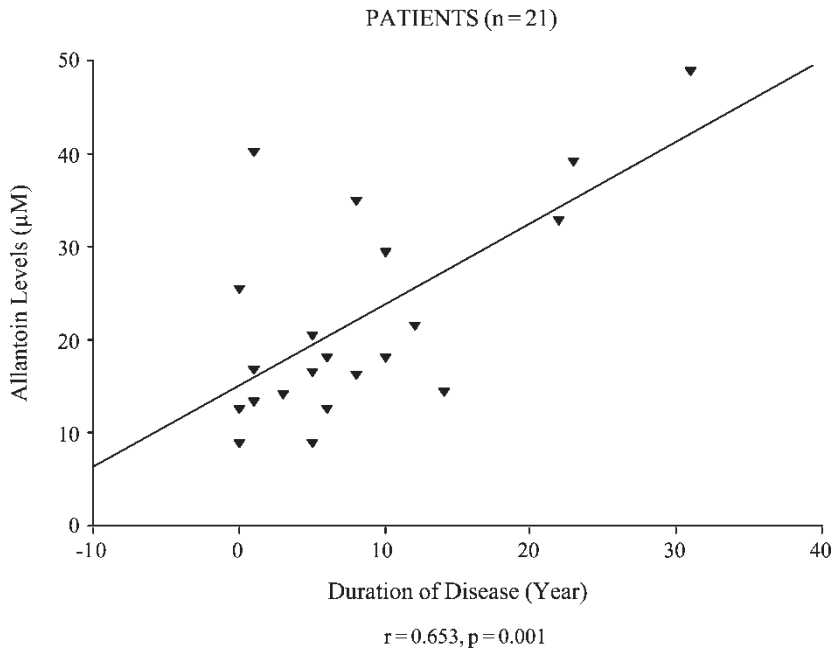

FIGURE 2 Correlations between allantoin and duration of disease in patients.

groups. This may be due to duration of exposure to free radicals produced in inflammation.

In a recent study, it was found that taking methotrexate, widely used as an anti-inflammatory drug in the treatment of rheumatoid arthritis, decreased serum uric acid levels.[43] Although we obtained lower levels of allantoin in patients who took no medicine, we could not find any effects of methotrexate or other medicines on the serum allantoin and uric acid levels.

In conclusion, our results imply that uric acid acts as a free radical scavenger and thus is converted to allantoin. Increased levels of allantoin, only a product of uric acid, support findings about elevated oxidative stress in rheumatoid arthritis.

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