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ORIGINAL ARTICLE

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# Methotrexate reduces the levels of pentosidine and 8-hydroxy-deoxy guanosine in patients with rheumatoid arthritis

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**Abstract** This study was performed to investigate whether methotrexate (MTX) affects the levels of oxidative stress markers, including pentosidine one of the glycation end products (AGEs) or 8-hydroxy-deoxy guanosine (8-OHdG). These stress markers represent DNA damage; 19 rheumatoid arthritis (RA) patients underwent MTX treatment. The levels of serum total, urinary total, urinary-free pentosidine and also urinary 8-OHdG, as well as clinical parameters, including disease activity scores for 28 joints (DAS28) were measured at baseline and at 3 and 6 months after the initial treatment with MTX. After the initial treatment with MTX, serum total and urinary total pentosidine levels were reduced at 6 months, and urinary-free pentosidine levels were reduced at 3 and 6 months. Urinary 8-OHdG levels also were significantly reduced at 6 months after the initial treatment with MTX. This study demonstrated that MTX plays a role as a regulator against pentosidine formation and oxidative DNA damage in RA patients.

**Key words** 8-Hydroxy-deoxy guanosine · Methotrexate · Pentosidine · Rheumatoid arthritis

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology and affects synovial joints, leading to joint destruction. A variety of disease-modifying anti-rheumatic drugs (DMARDs) are available to control the inflammatory process in RA. Methotrexate (MTX) has been widely used as the standard DMARD for the treatment of RA and malignancy.[1] MTX inhibits enzyme dihydrofolate reductase and acts as a cytotoxic drug.[2]

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It has been reported that MTX administered to rats increases malondialdehyde levels (an index of lipid peroxidation) but decreases the activity of superoxide dismutase, catalase, and glutathione peroxidase in renal tissue.[3] MTX induces oxidative stress[3] and causes oxidative damage in rat kidney tissue.[4] MTX could induce caspase-dependent apoptosis and promote in generating reactive oxygen species (ROS) along with disrupting the mitochondrial membrane potential of HL-60 and Jurkat T cells.[5]

A contradictory report on the effect of MTX, however, showed that ROS production induced by interleukin (IL)-6 in synovial cells from RA patients was suppressed by MTX.[6] One of the mechanisms of the anti-inflammatory and immunosuppressive effects of MTX has been suggested to depend on the reduction of the ROS production.

Reactive oxygen species causes tissue damage and is associated with pathophysiology in RA.[7,8] Large amounts of ROS have been detected in the synovial fluid of inflamed rheumatoid joints.[9] Pentosidine is one of the advanced glycation end-products (AGEs) formed under oxidative conditions.[10] It has been known that 8-hydroxy-deoxy guanosine (8-OHdG) is a product generated from oxidative DNA damage by oxygen radicals.[11] Oxidative stress including ROS is considered to be able to cause DNA damage and accelerate oxidation in the glycosylation processes of reduced sugar. Here, we have investigated changes in oxidative stress markers levels in RA patients treated with MTX.

In this study, we measured pentosidine and 8-OHdG levels in RA patients treated with MTX and evaluated the effects of MTX on the oxidative pathway in RA.

## Materials and methods

### RA patients receiving MTX treatment

In this study, informed consent in accordance with the guidelines of the ethics committee of Hamamatsu University School of Medicine was obtained from all participants;

19 patients (18 women and 1 man; mean age 58.4 ± 11.9 years; mean disease duration 11.2 ± 7.6 years; Steinblocker's stages I: 3, II: 4, III: 9, IV: 3) with RA, diagnosed according to the criteria of the American College of Rheumatology, were evaluated in this study between October 2004 and December 2006. We evaluated the patients administered with MTX for more than 6 months; 13 patients received a constant dosage of prednisolone (mean dosage 1.8 ± 2.3 mg/day) throughout this study; 16 patients received MTX at a constant mean dosage of 5.1 ± 1.5 mg/week (4–8 mg/week) for more than 6 months.

### Clinical and laboratory evaluation

Clinical and laboratory examinations including erythrocyte sedimentation rate/h (ESR/h), C-reactive protein (CRP), anti-agalactosyl IgG antibody (CA-RF), and a Disease Activity Score for 28 joints (DAS28) were carried out at the baseline and at 3 and 6 months after the initial treatment. The serum samples of RA patients were obtained just before an initial injection of MTX at the baseline and at 3 and 6 months after the initial treatment with MTX and were stored at −80°C until assayed. Serum anti-agalactosyl IgG antibody (CA-RF) was also measured with an ELISA kit (Ei test, Eisai, Tokyo, Japan). The urinary deoxypyridinoline (DPD) levels were measured by high performance liquid chromatography as described by Takahashi et al.[12] Serum bone alkaline phosphatase (BAP) levels were measured using an Alkphase-B kit (EIA: Metra Biosystems, Mountain View, CA, USA). The levels of serum total pentosidine, the urinary total and free pentosidine, and urinary 8-OHdG were measured in all the patients, as described below.

### Measurements of serum and urinary pentosidine levels

The levels of serum total, urinary total, and urinary-free pentosidine in RA patients before and after treatment with MTX and in control healthy individuals were measured by high-performance liquid chromatography, as described by Takahashi et al.[10] earlier. The total pentosidine in serum and urine is composed of protein-binding and free forms. The total pentosidine was measured after the pretreatment with acid hydrolysis, and free pentosidine was measured without pretreatment.

### Measurements of urinary levels of 8-OHdG

The urinary 8-OHdG levels from RA patients treated with MTX were measured with a competitive ELISA kit (8-OHdG check, Japan Institute for Aging, Shizuoka, Japan) according to the manufacturer's instructions. In this kit, 8-OHdG concentrations were measured at an absorbance of 450 nm. The levels of creatinine in urine samples were simultaneously measured for compensation of urinary levels 8-OHdG.

### Statistical analysis

All data are expressed as mean ± SD. The values from pre- and post-treatment measurements of pentosidine, 8-OHdG, CRP, ESR, serum CA-RF, DAS28, the number of swelling joints, and the number of tender joints were compared using the Wilcoxon signed rank test. Single regression analysis of these data was performed, and the statistical significance of correlation was determined with Pearson's correlation test. $P$ values less than 0.05 were considered significant. Statistical analysis in this study was performed on a Macintosh computer using Statcel software.

## Results

### Clinical and laboratory disease activity

CRP levels significantly decreased from 3.80 ± 2.98 mg/dl at pretreatment to 1.18 ± 1.25 mg/dl ($P < 0.01$) and 0.57 ± 0.34 mg/dl ($P < 0.01$) at 3 and 6 months, respectively, after the initial injection of MTX (Table 1). The ESR levels, DAS28, number of swelling joints, and number of tender joints also were significantly decreased at 3 and 6 months as compared with pretreatment. The serum CA-RF levels were significantly decreased at 6 months after the initial MTX treatment. The serum BAP levels did not show a significant difference during the follow-up periods. The urinary DPD levels were decreased at 3 and 6 months after initial treatments with MTX, as previously described in our reports.[13]

### Measurement of serum total and urinary total and free pentosidine

In this study, the levels of serum total, urinary total, and urinary-free pentosidine were measured. The mean levels of serum total pentosidine showed a significant change at 3 months (132 ± 41 nmol/l, $P < 0.05$) and at 6 months (133 ± 53 nmol/l, $P < 0.05$) compared with those at pretreatment (196 ± 102 nmol/l) (Table 1). The mean levels of urinary total pentosidine were significantly decreased at 6 months (3.9 ± 1.2 nm/mmol cre) compared with those at pretreatment (5.9 ± 3.5 nm/mmol cre). The mean levels of urinary-free pentosidine were also significantly decreased at 3 and 6 months after initial treatment with MTX. Because it has been reported that pentosidine levels are also raised in patients with renal dysfunction and diabetes mellitus, the serum levels of creatinine and glucose were also measured in obtained samples. These were in the range of normal values (data not shown).

### Measurement of urinary 8-OHdG levels

Urinary 8-OHdG levels were corrected with urinary creatinine levels. The mean urinary 8-OHdG levels showed a significant change at 6 months (8.8 ± 5.6 ng/mg cre, $P < 0.05$)

**Table 1.** Changes in laboratory and clinical parameters in patients with rheumatoid arthritis (RA) treated with methotrexate (MTX) and the laboratory parameters in normal controls

| | MTX | | | |
|---|---|---|---|---|
| | Baseline | 3 months | 6 months | Control |
| Serum total pentosidine (nmol/l) | 196 ± 102 | 132 ± 41* | 133 ± 53* | 82 ± 15 |
| Urinary total pentosidine (nmol/mmol cre) | 5.9 ± 3.5 | 5.0 ± 1.9 | 3.9 ± 1.2** | 3.8 ± 1.5 |
| Urinary-free pentosidine (nmol/mmol cre) | 5.4 ± 3.2 | 4.8 ± 1.9* | 3.3 ± 0.9** | 3.4 ± 1.4 |
| Urinary 8-OHdG (ng/mg cre) | 17.9 ± 9.6 | 7.5 ± 5.2 | 8.8 ± 5.6* | 8.6 ± 2.8 |
| CRP (mg/dl) | 3.80 ± 2.98 | 1.18 ± 1.25** | 0.57 ± 0.34** | ND |
| ESR (mm/h) | 71.5 ± 26.4 | 44.9 ± 24.4* | 33.5 ± 20.1** | ND |
| Serum CA-RF (AU/ml) | 402 ± 639 | 313 ± 435 | 316 ± 536* | ND |
| DAS28 | 5.23 ± 0.70 | 4.33 ± 0.73* | 4.09 ± 0.70* | ND |
| Number of swelling joints | 7.09 ± 3.18 | 5.73 ± 3.41* | 3.88 ± 2.03* | ND |
| Number of tender joints | 2.36 ± 2.20 | 0.67 ± 0.82 | 0.75 ± 0.87* | ND |
| Serum BAP (U/l) | 31.2 ± 16.6 | 36.6 ± 16.8 | 28.0 ± 10.5 | ND |
| Urinary DPD (nmol/mmol cre) | 14.2 ± 14.1 | 12.3 ± 10.1* | 9.8 ± 12.0* | ND |

Data are shown by mean ± SD values
ND, not done; CRP, C-reactive protein; ESR, erythrocyte sedimentation; DAS28, disease activity scores for 28 joints; BAP, bone alkaline phosphatase; DPD, deoxypyridinoline; 8-OhdG, 8-hydroxy-deoxy guanosine
** $P < 0.01$ versus baseline; * $P < 0.05$ versus baseline

**Table 2.** Single regression analysis of serum total, urinary total and urinary-free pentosidine; urinary 8-OHdG; other laboratory parameters; and DAS28 was performed, and the statistical significance of correlation determined with Pearson's correlation test

| | Serum total | Urinary total pentosidine | Urinary-free pentosidine | Urinary 8-OHdG |
|---|---|---|---|---|
| DAS28 | 0.443* | 0.755*** | 0.524* | 0.159 |
| Number of swelling joints | 0.327 | 0.156 | 0.160 | 0.219 |
| Number of tender joints | 0.200 | 0.522* | 0.572** | 0.229 |
| CRP | 0.581** | 0.499* | 0.499* | –0.090 |
| ESR/h | 0.453* | 0.333 | 0.328 | 0.099 |
| Serum CA-RF | –0.249 | –0.143 | –0.098 | 0.341 |
| Serum BAP | –0.248 | –0.157 | –0.133 | –0.002 |
| Urinary DPD | 0.278 | 0.755*** | 0.810*** | –0.026 |

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$

after the initial treatment with MTX compared with the levels at the initial treatment (17.9 ± 9.6 ng/mg cre).

### Correlation analysis among laboratory and clinical parameters

Serum total pentosidine levels significantly correlated with DAS28 (the correlation coefficient 0.443, $P < 0.05$), CRP (correlation coefficient 0.581, $P < 0.01$), and ESR/h (correlation coefficient 0.453, $P < 0.05$). Urinary total pentosidine levels significantly correlated with DAS28 (correlation coefficient: 0.755, $P < 0.0001$), the number of tender joints (correlation coefficient 0.522, $P < 0.05$), and CRP (correlation coefficient 0.499, $P < 0.05$).

Urinary-free pentosidine levels significantly correlated with DAS28 (correlation coefficient 0.524, $P < 0.05$), the number of tender joints (correlation coefficient 0.572, $P < 0.01$), and CRP (correlation coefficient: 0.499, $P < 0.05$). Urinary 8-OHdG levels did not show a significant correlation with the clinical and laboratory data measured in this study. However urinary total (correlation coefficient 0.755, $P < 0.0001$) and free pentosidine levels (correlation coefficient 0.810, $P < 0.0001$) correlated with the urinary DPD levels.

## Discussion

In this study, we demonstrated that MTX reduced the levels of pentosidine and 8-OHdG, the generation of which is considered to be associated with ROS production.

Many pharmacological actions of MTX have been described, including inhibition of the production of pro-inflammatory cytokines, regulation of lymphocyte proliferation and neutrophil chemotaxis and adherence, and reduction of serum immunoglobulin and the production of rheumatoid factors.[14] Low dosages of MTX also induce the apoptosis of mitogen-activates CD4+ and CD8+ T cells (but not resting T cells).[15,16] As an ex vivo action, MTX stimulates soluble tumor necrosis factor (TNF) receptor production, which might regulate the TNF action.[17] Furthermore, MTX inhibits the chemotaxis of neutrophils, which is considered to be the origin of ROS production.[18] MTX increases adenosine production, leading to an increased cyclic adenosine monophosphate (cAMP) level. High levels of cAMP cause decreased TNF-α and IL-6 production.[19] IL-1, IL-6, and TNF-α have been suggested to induce ROS production. MTX inhibits IL-1 and IL-6 activity in animal models.[14,18] On the other hand, MTX increased ROS production in the kidneys and liver, possibly damaging them.[3,4]

Reactive oxygen species stimulates chondrocytes and synovial fibroblasts to secrete matrix-metalloproteinases[20] and causes synovitis and destruction of the joints of RA.[7,8,21,22] $H_2O_2$ and superoxide ($O_2$–) has been shown to accelerate bone resorption by osteoclasts.[23,24] Some cytokines have been demonstrated to induce ROS production. For instance, IL-6 induces production of ROS in RA synovial fibroblastic cells.[6] TNF-α can cause ROS generation[25,26] in inflammatory arthritis such as RA and can induce ROS production from neutrophils. ROS affects the DNA synthesis pathway or the glycation cascade and induces the production of 8-OHdG and pentosidine. We measured the levels of urinary 8-OHdG, serum and urinary pentosidine in RA patients treated with MTX, and saw increased levels of pentosidine in the serum and urine at the baseline before treatment with MTX.

Pentosidine is one of the advanced AGEs formed under oxidative conditions and is formed non-enzymatically during spontaneous reaction of pentose with free amino acid composed of lysine and arginine and plays a role in the pathophysiology of RA.[27,28] The source of pentosidine production is glycosidation and oxidative stress, which may be mainly associated with pentosidine production in RA. HOCl is also produced by neutrophils and leads to the generation of dicarbonyls or pentoses, which produce pentosidine in the last stage.[29] Circulating serum AGEs are toxic and cause a pathological focus by reacting with tissue proteins; they induce the production of cytokines and can cause microangiopathy by binding to specific receptors in endothelial cells and macrophages.[29]

The function of pentosidine in RA patients remains obscure, but there are some data indicating that it has a pathological role in RA. But it remained to be determined which of the serum or urine samples is essential to evaluate the change of total pentosidine levels. The levels of urinary total and free pentosidine did not show a significant difference. Therefore the measurement of free pentosidine levels may be appropriate because it can be performed without acid hydrolysis procedure if there is no protein leak to urine by renal damage. Serum and urinary pentosidine levels are higher in RA patients than in age-matched OA patients, as we previously described.[30] The binding of AGE on receptors for AGE (RAGE) causes an activation of nuclear kappa B (NF-κB) and induces the production of proinflammatory cytokines.[31,32] IL-6 plays a role in osteoclast recruitment and activation. Hein et al. reported a significant correlation between serum IL-6 and serum pentosidine levels.[33] Takagi et al.[34] demonstrated that AGE enhanced the production of IL-6 in normal human bone-derived cells.[34] Other reports have shown that AGEs induce the production of cytokines, including IL-1, TNF-α, and insulin-like growth factor I in macrophages.[35,36] AGE-RAGE-mediated NF-κB activation is considered to be long-lived and to perpetuate inflammation. In addition, AGEs themselves induce oxidative stress and, as a result, activate NF-κB in vascular endothelium.[37]

In the present study, we measured the levels of serum and urinary pentosidine in RA patients treated with MTX. Interestingly, in RA patients at 6 months after initial treatment with MTX, serum total, urinary total, and urinary-free pentosidine levels were decreased. In particular, these levels were significantly correlated with the data representing disease activity, such as DAS28, and CRP. We may suggest, therefore, that the measurement of pentosidine can be useful in evaluating rheumatoid disease activity. However, urinary total and free pentosidine levels have shown a significant correlation with DPD, which has been considered to be a bone absorption marker. Higher pentosidine levels in diabetes mellitus patients have already been indicated.[38] Diabetes patients often have shown osteoporosis and higher urinary levels of DPD.[39] In the next study, we will study whether pentosidine has a function of bone absorption.

Furthermore, we have shown here that urinary 8-OHdG levels in RA patients treated with MTX were significantly decreased. Schraufstatter et al.[40] have demonstrated that hydroxyl radicals formed by $H_2O_2$, but not by HOCl, cause oxidative damage to DNA. When DNA is injured by ROS, guanine is changed to 8-oxo-guanine. Finally, 8-oxo-guanine is changed to 8-OHdG in the repair processes of DNA, and is excreted in the urine.[41,42] Urinary 8-OHdG has been considered to be a biomarker that can be used to indicate the extent of repair of ROS-induced DNA damage.[43–45] In the present study, we newly demonstrated that MTX reduced 8-OHdG levels of oxidative stress markers.

Pentosidine and 8-OHdG are later metabolites produced by oxidative stress. Our next study should be performed to measure directly the change in the levels of ROS production in RA patients treated with MTX.

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