

INCREASED URINARY NEOPTERIN : CREATININE RATIO AS A MARKER OF ACTIVATION OF CELL-MEDIATED IMMUNITY AND OXIDATIVE STRESS IN THE IRANIAN PATIENTS WITH MULTIPLE SCLEROSIS

H. Khorami,¹ TR. Neyestani,² Mehri. Kadkhodae,³ and J. Lotfi⁴

¹Department of Physiology, Faculty of Para-medicine, Shaheed Beheshti University of Medical Sciences, Tehran, Iran;² Laboratory of Nutrition Research, National Institute of Nutrition Research and Food Technology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran;^{3,4} Department of Physiology and Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

ABSTRACT

Neopterin, a pyrazinopyrimidine compound, is produced by macrophages after induction by interferon gamma (IFN- γ) and serves as a marker of cellular immune system activation followed by oxidative stress. The aim of this study was to determine urinary neopterin to creatinine ratio (UNCR) as a surrogate marker of cell-mediated immune activation in multiple sclerosis (MS). Three weekly early morning urine samples were collected from 27 patients with MS and 31 age- and sex-matched apparently healthy subjects. Urinary neopterin and creatinine were determined using reversed phase high-performance liquid chromatography and Jaffe reaction, respectively. UNCR was significantly higher in patients than in healthy controls indicating IFN- γ -induced cellular immunity activation and oxidative stress in multiple sclerosis. As a non-invasive method, UNCR determination may be helpful in monitoring disease progression and the effects of therapies, as well.

Keywords: Multiple Sclerosis, Neopterin, Cell-Mediated Immunity.

INTRODUCTION

Neopterin, a pyrazinopyrimidine compound, is produced by macrophages after induction by interferon gamma (IFN- γ) and serves as a marker of cellular immune system activation.¹ Large amounts of neopterin are released from human monocytes / macrophages mostly upon stimulation with IFN- γ ,² a typical Th1-type cytokine.

In humans, increased concentrations of neopterin in serum and urine have been found in viral infections including human immunodeficiency virus (HIV) type 1, various malignant disorders, autoimmune diseases such

as rheumatoid arthritis, and during allograft rejection episodes.³⁻⁷ Significant associations between enhanced neopterin and IFN- γ production have also been obtained in some other diseases,⁸ and monitoring neopterin concentrations has turned out to be a sensitive and useful marker for monitoring the activation of cellular (mostly of Th1-type) immune response.³ It has been shown that the amount of neopterin secreted by human monocytes /macrophages upon stimulation with IFN- γ correlates positively with the capacity of the same cells to produce reactive oxygen species.⁹ Likewise, in vivo neopterin concentrations were found to correlate on one hand with oxidation products of proteins¹⁰ and on the other hand with loss of some antioxidants like vitamin E.^{11,12} In summation, neopterin concentrations can also be regarded as an indicator for oxidative stress due to immune activation.^{13,14} Such an immunity

*Corresponding author: Dr. Tirang R. Neyestani, Laboratory of Nutrition Research, National Institute of Nutrition Research and Food Technology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran, P.O. Box 19395-4741.

hyperactivation may occur in many immune-mediated disorders such as multiple sclerosis (MS).¹⁵ Monocytes and macrophages appear to play important roles in MS, possibly related to their sensitivity to IFN- γ , which induces expression of class II human leukocyte antigens (HLA-DR, -DP, and DQ) and some other surface antigens and activates macrophages.¹⁶ The aim of this study was to assess urinary neopterin/creatinine ratio as a possible marker of IFN- γ -mediated inflammation and hence oxidative stress in the Iranian patients with MS.

Materials and Methods

Subjects

A case-control study was conducted with 27 patients of both sexes with relapsing-remitting MS from the Neurological Ward, Dr. Ali Shariati Hospital, Tehran, and 31 age- and sex-matched apparently healthy volunteers as controls attending the Department of Physiology, School of Medicine, Tehran University of Medical Sciences, Tehran. Those subjects, who had active infections based on history and clinical findings, were omitted from the study.

Procedures

Three weekly early morning urine samples were collected from each subject. Because of the sensitivity of neopterin to light and temperature, all samples were kept in dark and low temperature (4°C) while transportation to the laboratory, and then kept at -80°C until the day of assay (not more than 8 weeks). To assay aromatic neopterin, reversed phase high-performance liquid chromatography (HPLC) (Waters 600E) with the column Nova-Pack C18 (3.9×300 mm), Sorensen buffer pH 6.4 as an eluent with the flow rate of 0.8 ml/min and fluorescence detector (exciting at 338 nm and emitting at 425 nm) (Waters 420 AC) were used. The whole procedure was done at 30°C.^{16, 23} Urine samples were centrifuged at 3500 g at 4°C for 10 minutes and then 100 μ L of the supernatant was transferred to a fresh clean tube and then diluted with 900 μ L of either 5.4 mM of disodium ethylene diamine tetra acetic acid (Na₂EDTA) in 15 mM Sorensen buffer or 1 mM potassium iodide solution. With the latter solution, all oxidizable neopterin content was oxidized. The Sorensen buffer-diluted samples were analyzed for aromatic neopterin while the oxidized samples were used to determine total neopterin (both aromatic and oxidizable neopterin). The range of linearity was determined by plotting a standard curve using different concentrations of standard D (+) neopterin (Sigma). The concentration of urinary neopterin was determined by an integrator (Waters 746) using external standards. Urinary creatinine was measured with a commercial kit (Zist-Shimi Co, Iran) based on Jaffe reaction.¹⁷ Aromatic and total urinary neopterin

to creatinine ratio (UNCR) was then calculated. Mean value for weekly samples was used for each subject. Data with and without normal distribution were analyzed with student t – test and Mann-Whitney U Wilcoxon test, respectively, using Windows/SPSS 98 statistical package.

RESULTS

The retention time for aromatic neopterin was 4 min 29 sec±2.8 sec (Fig 1). The inter-assay variation in ten consecutive days was about 1%. The standard curve was linear up to the highest concentration of the standard neopterin, i.e., 1976 nM (Fig 2). Since the distribution of UNCR was not normal, and it could not be normalized using various methods of changing the variables, the data were analyzed with non-parametric Mann-Whitney U Wilcoxon test. Both aromatic and total UNCR were significantly higher in patients than in healthy controls (Table I). However,

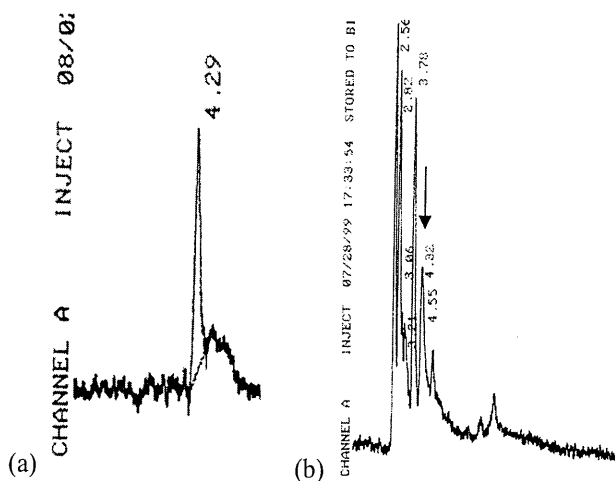


Fig. 1. (a) Chromatogram of the standard D(+) neopterin, retention time: 4.29"; (b) Chromatogram of a urine sample of a patient with multiple sclerosis, retention time: 4.32".

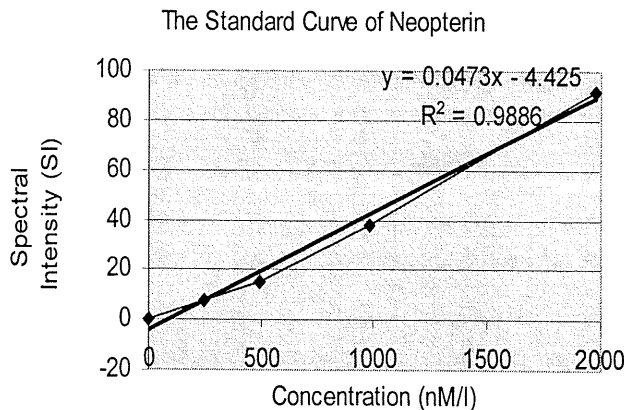


Fig. 2. Standard curve of neopterin. (See text for further details).

Table I. Aromatic and total UNCR in the subjects under study.

Group	Age ^a	Aromatic UNCR ^b	Total UNCR ^b	Aromatic to Total UNCR Ratio ^a
Patients with Multiple Sclerosis	33.9±1.4	1873.2±682.6 (900.0)	3309.7±1169.4 (1475.0)	0.67±0.05
Healthy Controls	30.8±1.9	307.9±44.9 (248.5)	473.0±60.0 (359.0)	0.60±0.06
Total (n=58)	32.3±1.2	1170.4±390.0 (518.0)	1868.1±599.6 (690.0)	0.64±0.04
P value	0.2	< 0.0001*	< 0.0001*	0.716

1. Values are mean±SEM. The numbers in parentheses show the medians.
2. UNCR: urinary neopterin to creatinine ratio
3. a: variables with normal distribution and analyzed with student t- test; b: variables without normal distribution and analyzed with Mann-Whitney U Wilcoxon test
4. * indicates significant difference between patients and controls in a column.

there was no significant difference in aromatic to total UNCR ratio between groups.

DISCUSSION

Increased UNCR in patients with MS indicates hyperactivation of the cellular immunity and oxidative stress as well. These days, the use of immunosuppressive drugs such as corticosteroids and cyclophosphamide is a routine treatment in MS. Alpha and beta interferons, which inhibit the synthesis of IFN- γ and reverse some of its immunostimulatory effects, may also be effective in preventing exacerbations.^{15,18} Considering the fact that increased UNCR indicates oxidative stress^{13,14} and overproduction of radicals caused by high levels of 7,8 dihydroneopterin may contribute to the pathogenesis of MS (19), some dietary manipulations may also prove to be helpful. In both approaches, i.e., drug or dietary treatments, response to therapy is highly dependent on the dose of the drug and/or supplement to be used, clinical stage, and the duration of supplementation among the other factors.^{18,19} To adjust the dose of the drug and/or supplement to be prescribed, it is very crucial to have a method to assess the immune activation in the course of the disease. As a non-invasive method, determining UNCR may prove to be useful in monitoring the effects of drug and/or dietary treatments. However, it must be taken into consideration that this marker is non-specific, so the results must be interpreted with caution.

The aromatic to total UNCR ratio has been reported

to be 45% in healthy subjects,¹⁶ though this ratio was considerably higher (~60%) in the Iranian subjects (both patients and healthy controls). This may partly be explained by popular use of iodinated salts by the Iranians, which may bring about oxidation of oxidizable neopterin in urine, increasing the amount of aromatic urinary neopterin, and hence increasing the aromatic to total UNCR. Our findings indicate that oxidizable neopterin is oxidized to the same extent in both patients with MS and healthy people.

In conclusion, UNCR is increased in patients with MS. Increased UNCR indicates activation of cellular immunity and oxidative stress. As a non-invasive method, UNCR assay may be used to monitor the effects of drug and dietary therapies. This latter concept needs further studies.

ACKNOWLEDGEMENTS

This work has been funded by Faculty of Medicine, Tehran University of Medical Sciences. All laboratory tests have been done at the Department of Physiology and Pharmacology, Faculty of Medicine, Tehran University of Medical Sciences.

REFERENCES

1. Fuchs D, Hausen A, Reibnegger G, et al: Neopterin as a marker for activated cell-mediated immunity: application in HIV infection, *Immunol Today*, 9(5):150-5, 1988.
2. Huber C, Batchelor JR, Fuchs D, et al: Immune response-

- associated production of neopterin. Release from macrophages primarily under control of interferon gamma. *J Exp Med*;160: 310-316, 1984.
3. Fuchs D, Weiss G, Reibnegger G, et al: The role of neopterin as a monitor of cellular immune activation in transplantation, inflammatory, infectious and malignant diseases. *Crit Rev Clin Lab Sci*; 29: 307-341, 1992.
 4. Baier-Bitterlich G, Wachter H, Fuchs D. Role of neopterin and 7,8-dihydroneopterin in human immunodeficiency virus infection: marker for disease progression and pathogenic link. *J Acquir Immune Defic Syndr* 13: 184-193, 1996.
 5. Reibnegger G, Fuchs D, Fuith LC, et al: Neopterin as a marker for activated cell-mediated immunity: application in malignant disease. *Cancer Detect Prev*;15: 483-90, 1991.
 6. Samsonov MY, Tilz GP, Egorova O, et al: Serum soluble markers of immune activation and disease activity in systemic lupus erythematosus. *Lupus*; 4: 29-32, 1995.
 7. Reibnegger G, Aichberger C, Fuchs D, et al: Post-transplant neopterin excretion in renal allograft recipients: reliable diagnostic aid of acute rejection and predictive marker of long-term survival. *Transplantation*; 52: 58-63, 1991.
 8. Fuchs D, Malkovsky M, Reibnegger G, et al: Endogenous release of interferon-gamma and diminished response of peripheral blood mononuclear cells to antigenic stimulation. *Immunol Lett*; 23: 103-108, 1989.
 9. Nathan CF. Peroxide and pteridine: A hypothesis on the regulation of macrophage antimicrobial activity by interferon-g. In: Gresser I, Vilcek J, editors, *Interferon*, Vol. 7, Academic Press, London, pp. 125-143, 1986.
 10. Witko-Sarsat V, Friedlander M, Nguyen Khoa et al: Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. *J Immunol*; 161: 2524-2532, 1998.
 11. Solichova D, Melichar B, Svobodova I, B. et al: Fluorescence analysis of antioxidant vitamins and neopterin in nonagenarians. *Biomed Chromatogr*; 13: 117-118, 1999.
 12. Sattler W, Leblhuber F, W. et al: Cerebrospinal fluid levels of a-tocopherol and neopterin in patients with dementia. *Pteridines*; 10: 220-224, 1999.
 13. Fuchs D, Baier-Bitterlich G, Wede I, et al: Reactive oxygen and apoptosis. In: *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, Scandalios J. (ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor-New York, pp. 139-167, 1997.
 14. Murr C, Fuith LC, et al: Increased neopterin concentrations in patients with cancer: indicator of oxidative stress? *Anticancer Res*; 19 (3A): 1721-1728, 1999.
 15. Amor S, Baker D, Layward L, et al: Multiple sclerosis: variations on a theme. *Immunol Today* 18: 368-71, 1997.
 16. Hausen A, Fuchs D, Wachter H. Determination of neopterin in human urine by reversed phase HPLC. *J Chromatography*; 27: 61-70, 1982.
 17. Slot C. Plasma creatinine determination. A new and specific Jaffe reaction method. *Scand J Clin Lab Invest* 17: 381-7, 1965.
 18. Karni A, Weiner HL. Multiple sclerosis. In: Rich RR, Fleisher TA, Shearer WT, Kotzin BL, Schroeder Jr. HW (eds.). *Clinical immunology, principles and practice*. 2nd ed., Mosby, London, pp: 77.8-10, 2001.
 19. Ergas D, Eilat E, Mendlovic S, et al: n-3 fatty acids and the immune system in autoimmunity. *Isr Med Assoc J*; 4: 34-8, 2002.