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Talanta 60 (2003) 459–465
Talanta
www.elsevier.com/locate/talanta

Fluorimetric determination of the levels of urinary neopterin and serum thiobarbituric acid reactive substances in the nonagenarians

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Received 17 June 2002; received in revised form 7 October 2002; accepted 25 November 2002

Abstract

Twelve self-sustaining nonagenarians, 10 women and two men, aged 94±3 years, and eight institutionalised nonagenarians, eight women, aged 91±1 year as well as 11 control subjects, seven women and four men, aged 84±5 years entered the study. Urinary neopterin, an indicator of systemic immune activation, and serum thiobarbituric acid reactive substances (TBARS), a marker of lipoperoxidation, were determined initially, and collection of the blood and urine samples was repeated at 3-month interval. Neopterin was measured in the urine specimens by reversed-phase high performance liquid chromatography. A C_{18} reversed-phase column 3.3 × 150 mm, 5 μm-diameter packing Separon SGX was used. Potassium phosphate buffer (15 mmol l^{-1}, pH 6.4) at flow rate of 0.8 ml min^{-1} was used as mobile phase. After centrifugation (5 min, 1300 × *g*) and diluting 100 μl of urine specimens with 1.0 ml of mobile phase containing 2 g of disodium–EDTA per litre, a 20 μl sample was injected on a column. Neopterin was identified by its native fluorescence (353 nm excitation, 438 nm emission). Creatinine was determined by Jaffé kinetic reaction after dilution of sample 1:50 (v/v). The concentration of neopterin in urine was expressed as neopterin/creatinine ratio (μmol mol^{-1} creatinine). TBARS were determined spectrofluorometrically using LS-5 spectrofluorimeter (excitation wavelength 528 nm, emission wavelength 558 nm) after extraction with *n*-butanol treatment with thiobarbituric acid. The significance of differences between nonagenarians and control group was examined by ANOVA–Kruskal–Wallis tests, using statistical software NCSS 6.0.21 (Kaysville, UT, 1996). The decision on significance was based on $P = 0.05$. Urinary neopterin was significantly higher in institutionalised compared to self-sustaining subjects and controls (625±565 vs. 203±63 μmol mol^{-1} creatinine, and 198±128 μmol mol^{-1} creatinine, respectively, $P = 0.006$). The serum TBARS were higher in both groups of nonagenarians (3.23±1.16 μmol l^{-1} and 2.69±0.39 vs. 2.12±0.83 μmol l^{-1} for the self-sustaining, institutionalised and controls, respectively, $P = 0.023$). We conclude that the

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0039-9140/03/$ - see front matter
doi:10.1016/S0039-9140(03)00073-0

fluorimetric determinations of urinary neopterin and serum TBARS can be useful for the monitoring health status in the elderly patients.

Keywords: Neopterin; Thiobarbituric acid reactive substances (TBARS); Elderly; Nonagenarians

1. Introduction

With aging, the population becomes more heterogeneous with regard to health status, due to the increasing prevalence of chronic illnesses and disability. Deterioration in health and functional status often induces important modifications in several biological parameters, including lipids. Among biochemical parameters usually used to assess the presence and the severity of specific diseases, lipid metabolites such as cholesterol, fatty acids and lipid peroxidation products have been long recognized as an indicators of general health, nutrition, and/or increased morbidity and mortality. The aim of the present study was the bioanalysis of urinary neopterin, an indicator of systemic immune activation [1], and serum thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation in the elderly patients and to evaluate the association of these biochemical parameters and health status.

2. Experimental

2.1. Reagents

2.1.1. Neopterin determination by HPLC

Deionized water and analytical-reagent grade chemicals were used. D-*erythro*-Neopterin (Fluka) was used for preparing external standard stock solution. 1.0 mg D-*erythro*-neopterin and 15.00 mg of dithiothreitol (Fluka) were dissolved in 850 ml deionized water in a volumetric flask. After 12 h mixing by magnetic stirrer and protected of light, the volume was diluted to 1000 ml by deionized water. Working solutions were prepared by diluting the 10 ml of the stock solution in 100 ml potassium phosphate buffer (15 mmol l^{-1}, pH 6.4). 1.0 ml of working solution was frozen and stored in −25 °C before HPLC analysis, stability of this working solution was 6 months.

Stock solutions of potassium phosphate buffer (15 mmol l^{-1}, pH 6.4) was prepared by dissolving 102.10 g dihydrogen potassium orthophosphate (Merck) and 57.10 g of hydrogen dipotassium orthophosphate (Merck) in 1000 ml deionized water in a volumetric flask and stored at 4 °C for 6 months. Fresh working solution was prepared from 15 ml stock solution diluting in volumetric flask 1000 ml deionized water before analysis.

2.1.2. Spectrofluorimetric analysis of TBARS

Thiobarbituric acid (TBA) reagent solution was prepared as fresh mixture 1:1 (v/v) saturated water solution of TBA (Fluka) and acetic acid (Merck) before analysis.

Ten percent phosphotungstic acid (Merck) was stored 1 week at reagent flask protected from light.

N-Butanol (Merck) and 0.034 mol l^{-1} sulphuric acid (Merck) were used for extraction TBARS and deproteinisation. 0.5 nmol l^{-1} standard water solution of malonyldialdehyde (Merck) was prepared fresh daily.

2.2. Apparatus

2.2.1. HPLC

System of high performance liquid chromatograph 1084 A (Hewlett-Packard, Palo Alto, USA), HP 79850 A integrator (Hewlett-Packard, Palo Alto, USA) and MPF-3 fluorescence detector (Perkin–Elmer, Norwalk, USA) were used, equipped with a C_{18} reversed-phase column 3.3×150 mm, 5 μm-diameter packing Separon SGX (Tessek, Praha, Czech Republic). A pH meter 525 A Orion (Boston, USA) was used for pH measurements.

2.2.2. Spectrofluorimetric analysis

LS-5 luminescence spectrometer (Perkin–Elmer, Norwalk, USA), centrifuge 400 R (Heraeus, Hanau, Germany) were used for this procedure.

2.3. Study group and design

Twelve self-sustaining nonagenarians, 10 women and two men, aged 94±3 years, followed at the Department of Metabolic Care and Gerontology, Charles University, Teaching Hospital and eight institutionalised nonagenarians, eight women, aged 91±1 year from Hospital for Chronically Ill as well as 11 healthy control subjects, seven women and four men, aged 84±5 years entered the study. None of the subjects was known to have cancer or an acute illness. The reason for institutionalisation was dependence because of immobility or deteriorating mental status. Self-sustaining nonagenarians had normal performance status and were considered to be nearly healthy (with exception of controlled hypertension or compensated diabetes mellitus). Urinary neopterin, an indicator of systemic immune activation, and serum TBARS, a marker of lipoperoxidation, were determined initially, and collection of the blood and urine samples was repeated at 3 months interval. All subjects gave informed consent to participate into the study and the local ethical committee, Charles University, Teaching Hospital, Hradec Králové, Czech Republic, had accepted the protocol of the study.

2.4. Procedure

2.4.1. Urinary neopterin analysis

Urine was collected between 06:00 and 09:00 h, protected from light and samples were stored at −25 °C before analysis. After centrifugation (5 min, $1300 \times g$) and diluting 100 μl of urine specimens with 1.0 ml of mobile phase containing 2 g of disodium–EDTA per litre, a 20 μl sample was injected on a column. D-*erythro*-Neopterin (Fig. 1) was measured in the urine specimens by reversed-phase high performance liquid chromatography [2,3]. Potassium phosphate buffer (15 mmol l^{-1}, pH 6.4) at flow rate of 0.8 ml min^{-1} was used as mobile phase. D-*erythro*-Neopterin was identified

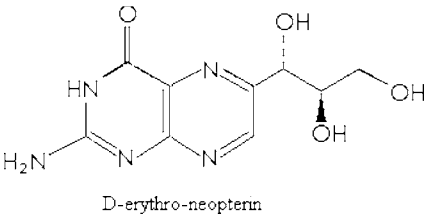

Fig. 1. Chemical structure of D-*erythro*-neopterin.

by its native fluorescence (353 nm excitation, 438 nm emission) (Fig. 2) and quantified by external standard method. The concentration of D-*erythro*-neopterin in urine was expressed as neopterin/creatinine ratio (μmol mol^{-1} creatinine). Creatinine was determined in the same sample specimen by Jaffé kinetic reaction after dilution of sample 1:50 (v/v) on a Hitachi 716 analyser (Tokyo, Japan) with commercial kit (Boehringer, Mannheim, Germany) according the manufacturer's instructions.

2.4.2. Spectrofluorimetric method for TBARS determination

Blood samples were drown from the peripheral vein after 12 h overnight fasting. After collection the samples were centrifuged, 10 min, $1400 \times g$, serum was separated and frozen and stored in −25 °C before analysis. Twenty microlitres of serum was analysed after extraction with *n*-butanol treatment with TBA, the modified method of Yagi [4] was used. TBARS were determined spectrofluorimetrically (excitation wavelength 528 nm, emission wavelength 558 nm).

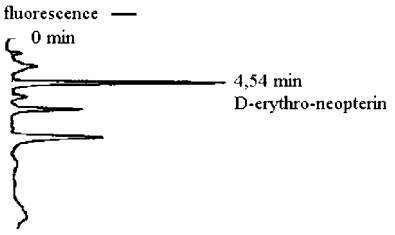

Fig. 2. Typical chromatogram of a human urine specimen with the peak of D-*erythro*-neopterin (194 μmol mol^{-1} creatinine, one patient from control group).

2.5. Statistical analysis

The significance of differences between nonagenarians and control group was examined by ANOVA–Kruskal–Wallis tests, using statistical software NCSS 6.0.21 (Kaysville, UT, 1996). Correlation between the variables was studied by Spearman rank correlation coefficient. The decision on significance was based on $P = 0.05$.

3. Results

The summary results of determination of urinary neopterin and serum TBARS in the elderly patients by fluorimetric detection techniques are shown in Table 1.

3.1. Urinary neopterin

Urinary neopterin was significantly higher in institutionalised nonagenarians compared to self-sustaining nonagenarians subjects and controls (625 ± 565 vs. 203 ± 63 μmol mol^{-1} creatinine, and 198 ± 128 μmol mol^{-1} creatinine, respectively, $P = 0.006$), Fig. 3. Urinary neopterin did not correlate significantly with serum TBARS.

3.2. Serum TBARS

The serum TBARS were higher in both groups of nonagenarians (3.23 ± 1.16 μmol l^{-1} and 2.69 ± 0.39 vs. 2.12 ± 0.83 μmol l^{-1} for the self-sustaining nonagenarians, institutionalised nonagenarians and controls, respectively, $P = 0.023$). These results are shown in Fig. 4. Serum TBARS exhibited

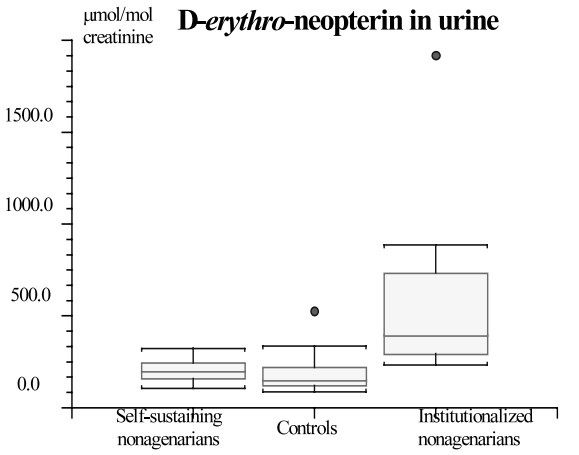

Fig. 3. D-*erythro*-Neopterin in urine (μmol mol^{-1} creatinine) in nonagenarians and controls.

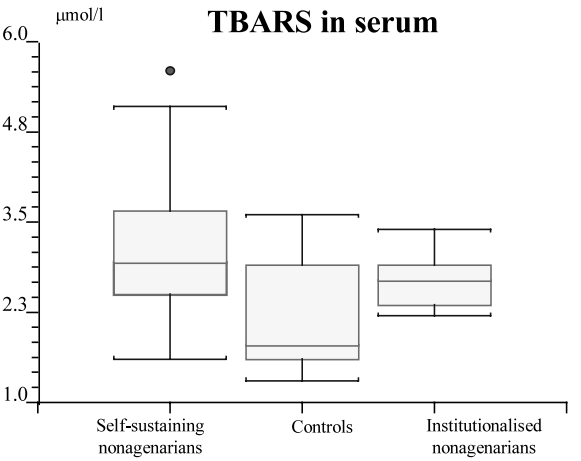

Fig. 4. TBARS (μmol l^{-1}) in serum of nonagenarians and controls.

Table 1
Urinary neopterin and serum TBARS in nonagenarians and controls

Subjects	*N*	S-TBARS (μmol l^{-1})		U-Neopterin (μmol mol^{-1} creatinine)	
		Mean (±SD)	Median	Mean (±SD)	Median
Nonagenarians self-sustaining	12	3.23 ± 1.16	2.95	203 ± 63	203
Nonagenarians institutionalised	11	2.69 ± 0.39	2.70	625 ± 565	397
Controls	8	2.12 ± 0.83	1.80	198 ± 128	155
P-value		0.023	0.006		

positive correlation with age (Spearman correlation coefficient 0.44, $P = 0.01$), Fig. 5.

4. Discussion

4.1. Urinary neopterin

In the present study we have observed markedly increased urinary neopterin levels in institutionalised nonagenarians compared to healthy nonagenarians and control subjects. This is in accord with an earlier study that demonstrated an association between urinary neopterin and mortality in the elderly [5]. The negative impact of increased neopterin concentrations on survival could be a reflection of a decline of immune function associated with high neopterin production, as demonstrated in cancer patients [6,7]. Data presented earlier by Solichová or Fahey [5,8] demonstrate that prognostic significance of neopterin may be observed in an aged population in general, although the design of the study did not make it possible to address the contribution of cancer-related mortality to the observed difference in neopterin excretion in survivors and non-survivors. Moreover, an increase in urinary neopterin concentrations in not restricted to cancer [9], but increased neopterin production is also observed in other conditions associated with systemic immune activation, including infectious diseases, autoimmune disorders [10] as well as atherosclerosis [11,12]. The association between increased neopterin levels and atherosclerosis, a condition responsible for a major part of morbidity and mortality in the elderly, might explain both the higher mortality observed in elderly individuals with increased neopterin levels [5] as well as increased urinary neopterin concentrations in institutionalised patients observed in the present study, as it is reasonable to assume that organ-specific manifestations of atherosclerosis were the cause of institutionalisation in many patients in the present cohort. Although we could only speculate about the causes of increased neopterin production in individual patients, occult cancers leading to deterioration in performance status are more likely to occur in institutionalised patients. Systemic immune activation and high neopterin production have also been demonstrated to be associated to anaemia [13], another possible cause of decline in performance status and institutionalisation. The prognostic significance of increased neopterin concentrations in the present cohort of institutionalised patients could not be examined because of the relatively small number of subjects. A study on a larger number of institutionalised patients could determine whether neopterin is a prognostic indicator for these patients.

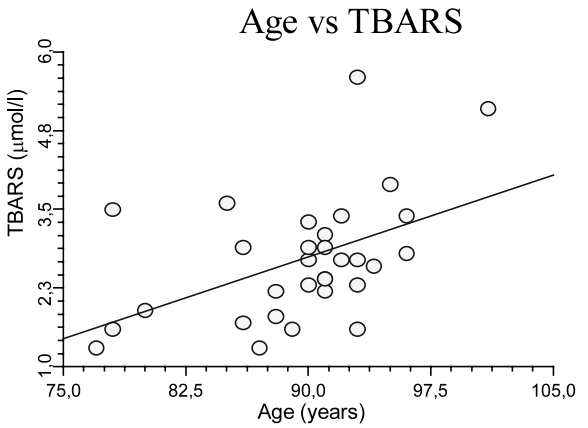

Fig. 5. Correlation of serum TBARS and age in all study groups.

Among different pteridines excreted in the urine, neopterin has been most widely studied in patients with different disorders, because of the association between neopterin production and systemic immune activation. Neopterin is produced from guanosine triphosphate (GTP) in a reaction catalysed by GTP cyclohydrolase I and is considered to be a relatively specific indicator of monocyte/macrophage activation [10]. When stimulated with interferon-γ, human monocytes release large quantities of 7,8-dihydro-neopterin [14], a precursor in the synthesis of 5,6,7,8-tetrahydrobiopterin. As monocytes lack enzymes in the synthetic pathway leading to the synthesis of 5,6,7,8-tetrahydrobiopterin distal from GTP cyclohydrolase I, 7,8-dihydroneopterin is released into the body fluid. 7,8-Dihydroneopterin is oxidized to neopterin that could be detected by its native fluorescence. It was demonstrated that concentrations of neopterin correlate with the concentrations of total neop-

terin (i.e. the sum of neopterin and 7,8-dihydroneopterin concentrations measured after oxidative treatment of the samples) [15], and neopterin concentrations are therefore routinely measured without oxidative pretreatment [9,10]. The avoidance of an oxidation step also simplifies the requirement for sample handling, making the method more practical.

Although an increase in urinary neopterin is accompanied by a decrease in biopterin concentrations, and the neopterin/biopterin ratio was used to discriminate cancer patients from controls in individual studies [16], neopterine/creatinine ratio has become universally accepted method of expressing neopterin concentrations in the urine [1,10]. Although present in urine in higher concentrations than neopterin, xanthopterin determination has not gained widespread use because xanthopterin concentrations may be dependent on factors other than systemic immune activation, e.g. liver injury [17].

The present HPLC method with fluorescent detection has gained widespread use because of relative simplicity and is suitable for both routine and investigational measurements. Other procedures described for measuring pteridines in biological fluids include, among others, derivative spectrophotometry [18] or liquid-chromatography/electrochemistry [19], chiral high performance liquid chromatography [20], capillary electrophoresis [21], but these methods have still not been widely used for clinical or investigational purposes.

4.2. TBA reactive substances

Numerous theories have been proposed to explain the association between old age and increased frequency of disorders [22]. Among the theories, cellular and tissue damage by oxygen free radicals is gaining prominence, and oxidative stress is thought to play an important role in the pathogenesis of disorders associated with aging [23]. The main product of fatty acid reaction with free radicals, malondialdehyde (MDA), reacts with TBA to give a product with fluorescence at 553 nm. The method of Yagi [4] was used in this study for the determination of TBARS—marker of lipid peroxidation and oxygen free radicals-induced damage. We reported previously [24] on significantly higher levels of serum TBARS in the group of nonagenarians independent of the health status. Thus, the age may be more important factor in determining the serum TBARS concentration than the presence or absence of a specific pathological condition. The correlation observed between age and serum TBARS is a hypothesis generating finding that should be confirmed in a more homogenous group of normal subjects. This observation is also in agreement with the report Carrera-Rotllan et al. [25,26] who demonstrated an increase of TBARS and other parameters during aging. Higher concentrations of TBARS in serum in the nonagenarians can be linked to higher concentrations of polyunsaturated fatty acids in LDL, which are substrates for oxidation [24,27]. No correlation was observed between urinary neopterin and serum TBARS, indicating that lipid peroxidation and systemic immune activation are independent biological phenomena in the studied population.

In conclusion, in the present study we observed the significantly higher levels of urinary neopterin and serum TBARS in the group of nonagenarians compared to the control group. These biochemical parameters determined by a fluorimetric analytic methods might be useful for monitoring health status in the elderly patients.

Acknowledgements

The authors want to thank Marie Burešová, Drahomíra Jánská, Marie Mejtská and Iveta Svobodová for excellent technical assistance. Supported by grants of Ministry of Health Czech Republic, N0. NG/6770-3, NC/6171-3 and NB/6822-3.

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