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Malondialdehyde and 8-oxo-7.8-dihydro-2'Deoxyguanosine in the Urine of Residents from Balkan Endemic Nephropathy Area in Croatia – A Pilot Study

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A B S T R A C T

Balkan endemic nephropathy (BEN) is a human chronic tubulointerstitial renal disease that occurs in rural areas of some Balkan countries. The disease is insidious and fatal, and mostly affects persons in their sixties or seventies. BEN areas have unusually high rates of otherwise rare upper urinary tract tumors (UTT). Since extensive production of reactive oxygen species leading to oxidative stress has been implicated in tumor development, the aim of this study was to see whether oxidative stress is involved in the development of BEN and UTT. Urine samples were collected from a BEN village (N=22) and a control village (N=16) residents and analyzed for malondialdehyde (MDA) and 8-oxo-7,8-dihydro--2'deoxyguanosine (8-oxodG). The levels of both oxidative stress parameters were significantly higher in the BEN village residents than controls. However, there was no correlation between MDA and 8-oxodG results. Our results confirm that oxidative stress could be implicated in development of both, BEN and UTT.

***Key words**: biomarkers of oxidative stress, healthy subjects, kidney, urinary tract tumors*

Introduction

Balkan endemic nephropathy (BEN) is a human chronic tubulointerstitial renal disease that occurs in rural areas of Croatia, Bosnia and Herzegovina, Bulgaria, Kosovo, Romania, and Serbia. The disease has insidious onset and fatal outcome, mostly in patients in their sixties and seventies who have lived in a BEN area for at least one decade[1]. The early signs of BEN are mild anemia without hypertension and proteinuria of the tubular type. In the advanced stage of BEN, severe fibrosis and atrophy develop, leading to end-stage renal disease and renal failure. In Croatia, the prevalence of BEN has been followed carefully from the early 1970s. In an epidemiological survey conducted in the spring of 2005, the prevalence of BEN was 0.6–2.3%, which is approximately the same as in the past decades, and which still makes this disease a serious health issue in Croatia[2].

High incidence of otherwise rare upper urinary tract tumors (UTT) in BEN areas was first noticed in Bulgaria and then in other countries[1,3]. These tumors are usually multiple and malignant, and localized mostly bilaterally in the renal pelvis, while the localization in the urinary bladder is not more frequent than in other areas. In 1995–2002, UTT mortality in the Croatian BEN area was 14 times higher than in the rest of the region and 55 times higher than in Croatia.[1] In spite of all the efforts to understand BEN and UTT, their etiology is still unknown. A number of more or less plausible hypotheses have been proposed. Since BEN and UTT appear in restricted geographical areas and exclusively in rural population, it is assumed that genetic factors and exposure to a natural toxic and carcinogenic compound should stand behind both diseases. Aristolochic acid (AA), ochratoxin A (OTA), and coal leachates have by now received most attention[4]. Molecular epidemiology supports the role of AA (nephrotoxic and carcinogen substance in Chinese slimming herbs), although causative roles of OTA and coal leachates have not been ruled out altogether[5].

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Many mutagens, tumor promoters and carcinogens are known to generate reactive oxygen species (ROS) leading to oxidative stress that may be involved in several mechanisms of carcinogenesis. Similary, several human chronic diseases are strongly related to oxidative stress[6,7]. Oxidative stress oxidizes macromolecules such as lipids, proteins, and DNA[8]. In this study urinary excretion of malondialdehyde (MDA), a marker of lipid peroxidation, and 8-oxo-7.8-dihydro-2'deoxyguanosine (8-oxodG), a marker of oxidative damaged guanine in DNA or in the nucleotide pool were compared between the residents of BEN area households and of non-BEN area households in Croatia. The hypothesis was that oxidative stress markers should be elevated in the first group, which is at increased risk for BEN and UTT in respect to general population, because oxidative stress is involved in the pathogenesis of both diseases.

Materials and Methods

Chemicals

Standards of 8-oxodG and MDA (1,1,3,3-tetramethoxy propane), thiobarbituric acid (TBA), and butylated hydroxytoulene (BHT) were purchased from Sigma Chemicals (St. Louis, MO, USA). Potassium chloride, phosphoric acid, acetonitrile, and methanol were from Kemika (Zagreb, Croatia) and potassium dihydrogenphosphate (KH_2PO_4) and water were from Merck (Darmstadt, Germany). All chemicals were of pro analysis grade. Water, acetonitrile, and methanol used for mobile phase were of HPLC grade.

Samples

Sample collection was approved by the Ethics Committee of the Institute of Public Health of the Republic of Croatia, and all participants gave informed consent. In spring 2007, spot morning urine samples were collected in a BEN village of Kaniža from apparently healthy individuals living in the household with present or past BEN history and at increased risk for BEN and UTT (BEN--residents; N=22). Control urine samples were collected from residents of non-BEN villages, but from the same area (controls; N=16). The volunteers of both groups had no hypertension, proteinuria, or alfa-microglobulinuria, anemia or increased blood creatinine. The urine samples were immediately centrifuged and the supernatants were divided into labeled tubes for different analysis and then stored at –80 °C until analyzed.

8-oxodG assay

8-oxodG was determined by use of HPLC with electrochemical detection. The urine samples were cleaned-up on a Bond Elut Certify cartridge (Varian, Harbor City, CA, USA).

HPLC consisted of a Varian ProStar 210 isocratic pump with pulse damper connected to a Varian ProStar 370 electrochemical detector (Walnut-Creek, CA, USA). HPLC separation was performed on a reverse-phase C18 column, 5 μm, 150 x 4.6 mm (Microsorb 100-5 BDS, Varian, Harbor City, CA, USA). The working electrode potential was set at +0.6 V. The analytical column and flow cell were kept in a Faraday-shielded oven compartment and temperature was set at 30 °C. Chromatographic data were collected and processed using Star Chromatography Workstation software (Ver. 5.0, Varian, Walnut-Creek, CA, USA). The mobile phase for determination of 8-oxodG contained: 50 mM of KH_2PO_4, 2 mM of KCl, 2.5% acetonitrile, and 1% methanol (pH 4.45), and a flow rate was set at 0.5 mL/min. 8-oxodG was quantified in the urine sample by peak-area measurement using the linear regression curve for aqueous 8-oxodG standard solutions (50, 100, 200, 400 nmol/L).

MDA assay

MDA measurement in urine was done according to the TBA assay on an HPLC consisted of a Varian INERT 9012 gradient pump and Varian 9075 fluorescent detector (Walnut-Creek, CA, USA). Separation of the MDA--TBA_{2-1} adduct was performed on an analytical column coupled with a 5 μm guard column (LiChrospher RP-18, Merck, Darmstadt, Germany) and their size was 125.0 x 4.0 and 4.0 x 4.0 mm, respectively. Detector excitation wavelength was set at 514 nm and emission at 544 nm. The analysis was performed at room temperature. Chromatographic data were collected and processed using Star Chromatography Workstation software (Ver. 5.0, Varian, Walnut-Creek, CA, USA). The mobile phase for MDA analysis contained 50 mM mixture of KH_2PO_4 and methanol (60:40) and a flow rate was 0.5 mL/min. As with 8-oxodG, for MDA quantification we used a calibration curve of aqueous standards.

Creatinine assay

Creatinine concentrations in urine were determined using standardized method.

Statistics

Data are presented as $\overline{X}\pm SD$. Results for 8-OHdG are expressed in nmol/L and μmol/mol creatinine, while MDA concentrations are expressed in μmol/L and mmol/mol creatinine. The Kolmorgorov-Smirnov test verified that the results were normally distributed. To test the significance of differences in urine MDA and 8-oxodG between BEN village and control residents, we used Student's t-test for two independent samples. Pearson's test was used to test the correlation between MDA and 8-oxodG. Statistical program Statistica 8.0 (Stat Soft Ltd., Bedford, UK) was used. Probability values of $p<0.05$ were considered statistically significant.

Results

We took urine samples in this study even though it is a more complex biological matrix for the analysis than blood, because collection is not invasive and was more readily accepted by the subjects. The study of Pilger et al.[9] demonstrated that 8-oxodG in 24-hour urine and in

TABLE 1
MDA AND 8-oxodG URINE CONCENTRATIONS IN BEN VILLAGE AND CONTROL VILLAGE RESIDENTS IN CROATIA. RESULTS ARE PRESENTED AS MEANS ± SD

Urine sample	MDA μmol/L (range)	MDA mmol/mol creatinine (range)	8-oxodG nmol/L (range)	8-oxodG μmol/mol creatinine (range)
Control village (N=16)	0.47±0.25 (0.14–0.92)	0.06±0.04 (0.02–0.16)	4.37±3.90 (0.8–25.6)	0.51±0.31 (0.0–1.17)
BEN village (N=22)	0.79±0.41 (0.27–1.70)*	0.13±0.09 (0.04–0.36)*	8.62±6.67 (0.0–14.4)*	1.42±1.18 (0.14–4.71)*

* significantly different from control group ($p<0.05$).

spot urine sample correlated reasonably well, and that spot urine contained relevant 8-oxodG concentrations. This is why we collected morning spot urine samples for our analysis.

Urine MDA concentrations are expressed in μmol/L and in mmol/mol creatinine, while 8-oxodG concentrations are expressed in nmol/L and μmol/mol creatinine. Both MDA and 8-oxodG findings in BEN village residents were significantly higher than in controls ($p<0.05$, Table 1).

Pearson's test demonstrated that MDA and 8-oxodG did not correlate ($p=0.1965$, Figure 1).

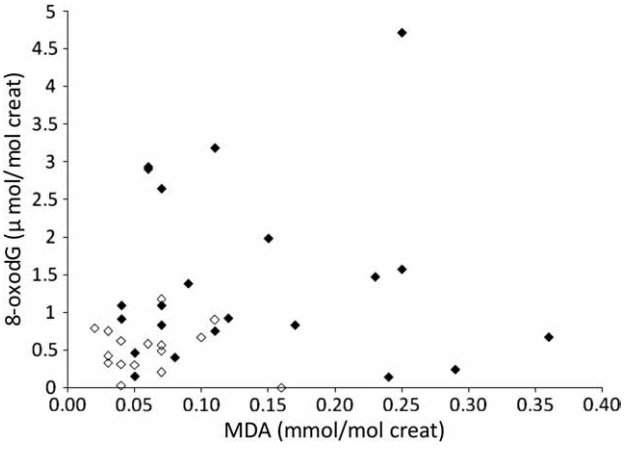

Fig. 1. Correlation between MDA and 8-oxodG urine concentrations in BEN village (♦) and control village residents (◇) in Croatia.

Discussion

This is the first study showing that Croatian BEN village residents have higher urine concentrations of deoxynucleotide and lipid oxidation products than persons living in a village without BEN. These findings put forward oxidative stress as one of the mechanisms of BEN and UTT development.

Our study is in line with genetic polymorphisms studied in a Bulgarian BEN area. Atanasova et al.[10] reported that the CYP3A5*1 allele, a marker for CYP3A5 expression in human kidney, was associated with increased risk for BEN (odds ratio 2.41). The authors suggested that the carriers of the CYP3A5*1 allele could activate xenobiotics that induce BEN and create individual predisposition for the disease. It is well known that cytochrome P450 monooxygenases, drug-metabolizing enzymes, play a major role in detoxification and elimination of hydrophobic xenobiotics. Paradoxically, these enzymes also generate ROS. It is possible that higher activity of CYP3A5 has toxic effects in BEN patients, not only because it induces production of carcinogen metabolites, but also because it increases ROS production.

A study by Andonova et al.[11] found an association between a wild-type allele that encodes glutathione S-transferase M1 (GSTM1) and BEN. GSTs are phase II enzymes, that are generally believed to be detoxifying, and their higher activity is involved in cell protection against xenobiotics and oxidative stress. It is established that active GSTM1 (encoded with wild-type allele) can react with phospholipid peroxides formed during oxidative stress and protect cell DNA from oxidative damage. Andonova et al.[11] has therefore postulated that higher activity of GSTM1 found in BEN patients may deplete GSH pool and that a ROS-producing xenobiotic that causes BEN cannot be conjugated due to the GSH depletion. It is the extent of GSH depletion and susceptibility of cell to ROS that could be crucial in BEN development. Our results together with results from the Bulgarian studies suggest that BEN is caused by one or more xenobiotics that can produce high level of ROS.

ROS are involved in multistage carcinogenesis. Numerous studies have proven that ROS activate redox-sensitive signaling molecules such as NF-κB and AP-1, and disturb the cell signaling and gene expression systems[12]. Activation of p53 is also considered a cellular response to oxidative stress. A series of studies have shown that ROS induce mutagenesis of hot spot codons (248 and 249) in the human p53 suppressor gene. p53 gene is an important tumor suppressor gene that maintains genome integrity and accuracy of chromosome segregation. More than 50% of human cancers contain mutations in the p53 gene.

Studies of biomarkers of carcinogenesis in biological material of persons from BEN areas are rather scarce. However, in a study of UTT patients living in a Croatian BEN area, Grollman et al.[13] found a high percentage of mutations, with AT to TA transversion in the p53 gene of carcinoma cells. This may seem to contradict our results, because 8-oxodG causes GC to TA transversion[14]. The reason however may be that we have measured parameters of oxidative stress in urine of healthy persons at risk,

while other authors have analyzed biological material (e.g. tumors) of patients with UTT at an advanced stage.

Both urine MDA and 8-oxodG are considered markers of »whole-body« oxidative stress[15]. Even so, Bergman et al.[15] found that their urine levels did not correlate, just as we did. Higher MDA and 8-oxodG were found in malignant diseases and were associated with carcinogenesis.[8] Loft et al.[16] associated high urinary excretion of 8-oxodG with increased risk of lung cancer among non-smokers and concluded that urine 8-oxodG could be an important biomarker of exposure to carcinogens and could predict cancer risk.

Oxidative stress plays an important role in cell regulation and can cause mutations and tumor. In this study we hypothesized that oxidative stress could be involved in the pathogenesis of BEN and UTT. BEN is a chronic disease, and the absence of an acute phase makes early diagnosis improbable. Hence there is a need for a reliable biomarker. This is the first Croatian study reporting a higher level of oxidative stress biomarkers in the urine of healthy individuals living in households with BEN history than in control population, which suggest that they may find proper use as early biomarkers of BEN and UTT.

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KONCENTRACIJA MALONDIALDEHIDA I 8-OKSO-7,8-DIHIDRO-2'-DEOKSIGVANOZINA U URINU STANOVNIKA IZ PODRUČJA BALKANSKE ENDEMSKE NEFROPATIJE U HRVATSKOJ – PRELIMINARNO ISTRAŽIVANJE

SAŽETAK

Balkanska endemska nefropatija (BEN) je kronična, fatalna, neupalna, tubulointersticijska bolest bubrega koja se pojavljuje u ograničenim, ruralnim područjima Bosne i Hercegovine, Bugarske, Hrvatske, Kosova, Rumunjske i Srbije. Od BEN obolijeva seosko stanovništvo starije životne dobi (u šezdesetima i sedamdesetima godinama). U endemskom području zamijećena je veća učestalost, inače vrlo rijetkih, epitelnih tumora gornjeg dijela mokraćnog trakta (UTT). Povećan nastanak slobodnih radikala i posljedični oksidacijski stres povezuje se s nastankom tumora te je stoga cilj ovoga istraživanja bio utvrditi povezanost oksidacijskog stresa s nastankom BEN i UTT. Uzorci urina sakupljeni su od zdravog stanovništva iz BEN sela (N=22) i kontrolnog sela (N=16) te je u njima određena koncentracija pokazatelja oksidacijskog stresa, malondialdehida (MDA) i 8-okso-7,8-dihidro-2'deoksiguanozina (8-oksodG). Koncentracije oba mjerena pokazatelja oksidacijskog stresa bile su značajno povišene u urinu stanovnika iz BEN sela nego iz kontrolnog sela. Korelacije između rezultata MDA i 8-oksodG nije bilo. Naši rezultati ukazuju da je oksidacijski stres uključen u nastanak i razvoj obje bolesti, i BEN i UTT.