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Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine and 5-(hydroxymethyl) Uracil in Smokers

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Cigarette smoke is known to generate free radicals by various mechanisms. In this study involving 30 non-smokers and 30 smokers, we show that urinary excretion of 5-(hydroxymethyl) uracil (HMUra) was not different in the two groups (6.54 ± 2.07 vs. 6.70 ± 1.68 nmol/mmol creatinine). In contrast, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) excretion increased by 16% (1.16 ± 0.35 vs. 1.35 ± 0.50 nmol/mmol creatinine, $p = 0.039$). Results concerning 8-oxo-dGuo are in agreement with those of previous studies. We observed significant multiple correlations between HMUra and creatinine ($r_p = 0.44$), BMI ($r_p = -0.27$) and nicotine derivatives ($r_p = 0.26$). Multiple correlation analysis showed relations between 8-oxo-dGuo on the one hand, and: creatinine ($r_p = 0.36$), nicotine derivatives ($r_p = 0.29$), BMI ($r_p = -0.24$) on the other.

Keywords: DNA damage, 5-(hydroxymethyl) uracil, 8-oxo-7,8-dihydro-2′-deoxyguanosine, cigarette-smoke, free radicals

INTRODUCTION

Cigarette smoke is a complex mixture of over 4,700 chemical compounds, including free radicals and other oxidants.[1,2] The gas phase of cigarette smoke contains nitric oxide, peroxinitrite, whereas quinones, semiquinones, and hydroquinones[3,4] are present in cigarette tar. Semiquinone can easily form superoxide radical while the parental quinone molecule is regenerated. Superoxide dismutase converts superoxide ions into hydrogen peroxide. Superoxide ion in the presence of hydrogen peroxide, and ferrous iron gives rise to the highly toxic •OH radical by the Haber–Weiss cycle. Peroxinitrite is highly toxic and can also generate •OH radicals. The production of •OH within the cell causes damage to DNA, membranes, and various cell structures that could explain tobacco induced carcinogenesis.[5]

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Hereby, we compare and discuss the measurement of two DNA oxidative lesions – urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) and 5-(hydroxymethyl) uracil (HMUra) – in 30 smokers comparatively to 30 non-smokers. Two previous studies had been devoted to the measurement of 8-oxo-dGuo in smoker urine.[6,7] However, the simultaneous measurement of two markers of DNA oxidation, in 24-h urine collections from smokers, has not been reported.

HMUra is formed by •OH radical attack on the methyl group of thymine.[8,9] HMUra is removed from DNA by 5-(hydroxymethyl) uracil DNA glycosylase,[10,11] eliminated from cells in blood, and eventually excreted in urine. HMUra and 5-(hydroxymethyl)-2′-deoxyuridine have been used as markers of cellular DNA damage by oxygen free radicals. In a previous study involving 14 patients it was shown that adriamycin treatment increased HMUra excretion by 30%.[12] Adriamycin forms •OH via a semiquinone mechanism, which may be similar to cigarette smoke, but, in cancer treated patients, semiquinone is received at much larger amounts (30 mg adriamycin/m^2 every month) than in mild smokers.

Besides HMUra, 8-oxo-dGuo is one of the most common oxidative DNA lesions,[13] and arises from C-8 hydroxylation of 2′-deoxyguanosine. Whereas hydroxyl radicals generate multiple products from all four bases, singlet oxygen preferentially alters 2′-deoxyguanosine, particularly through 8-hydroxylation.[14] *In vivo*, damaged nucleotides are released from nucleic acids by exonucleases and nucleotide-excision-repair. Thereafter, these nucleotides may form nucleosides after dephosphorylation by 5′-nucleotidase.[15] On the other hand, specific DNA glycosylases release altered bases. These repair products are then excreted in urine. Thus 8-oxo-dGuo has been proposed as a biomarker of oxidative DNA damage and repair.[16] The base 8-oxo-7,8-dihydroguanine (8-oxo-Gua) can be repaired by Fapy glycosylase.

MATERIALS AND METHODS

Chemicals

HMUra was from Sigma (St Louis, MO, USA). *N*-(*tert*.-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (MTBSTFA) from Fluka (Mulhouse, France) was used to prepare volatile GC derivatives. Water for HPLC was purified with a Milli-Q system (Millipore, Milford, MA). The internal standard for GC-MS was [1,3-$^{15}N_2$,5-$^{2}H_2$]HMUra. It was synthesized from [1,3-$^{15}N_2$]uracil and [$^{2}H_2$]paraformaldehyde (Sigma).[17]

Materials

HPLC Semipreparative Apparatus

A Kontron system (Kontron Analytic, Montigny-le-Bretonneux, France) was used. It was made up of two 420 pumps, a Kontron 460 autosampler, and a 450 data system to control pump flow and calculate concentrations. A Shimadzu SP6AV UV/Visible (Shimadzu Electronics, Tokyo, Japan) detector monitored the column output. For HMUra measurements, semipreparative HPLC was performed with a 250 mm × 7.5 mm i.d. stainless steel column that was packed with Spherisorb ODS2, 5 μm particle size. Urine samples were eluted with water at 2.5 ml/min. After each run, the column was recycled with acetonitrile/water (60 : 40, v/v). For 8-oxo-dGuo measurements, urine samples were purified on a 250 mm × 10.0 mm i.d. stainless steel column packed with an Alltech Adsorbosphere C18 HS stationary phase. Samples were eluted with a 5 mM phosphate buffer adjusted at pH 7 which contained 2.4% acetonitrile. Pump flow was set at 4.4 ml/mm. Components of the mobile phases were first filtered through Millipore 0.45 μm filters and degassed under vacuum. Fractions that contained either HMUra or 8-oxo-dGuo were collected with a Pharmacia FRAC-100 fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Gas Chromatograph-Mass Spectrometer

Quantitative determination of HMUra was achieved with a ThermoQuest TRIO 1000 gas-chromatograph mass-spectrometer (Les Ulis – France). The gas-chromatograph was equipped with a 25 m × 0.32 mm I.D. Chrompack CP Sil five column (Chrompack, Les Ulis, France) internally lined by a 1.2 µm film. Derivatized extracts of samples and standards were injected onto the column using a splitless injector.

HPLC/EC Apparatus

8-Oxo-dGuo was measured with a Kontron HPLC system consisting of a 422 pump, a 360 auto-sampler, and a ESA Coulochem II coulometer equipped with a 5011 analytical cell. Separations were performed on an Alltech Alltima C18 column (250 mm × 4.6 mm i.d.) eluted with a 20 mM phosphate buffer adjusted at pH 3 and containing 5% acetonitrile.

Other Apparatus

A Hitachi 917 autoanalyser (Boehringer-Mannheim France S.A., Meylan, France) was used to measure creatinine in urine.

Biochemical Determinations

HMUra was determined in 24-h urine collections by GC-MS and isotopic dilution using a method that we had previously described.[18] Port temperature was set at 230°C and gas flow rate in the column was 1.5 ml/min. At the end of each chromatographic run, after the injection oven temperature was reached, a 3 min delay was waited before the injection of the next sample.

8-Oxo-dGuo was measured in 24-h urine collection by HPLC/EC according to a previously reported method.[19] 8-Oxo-dGuo was extracted from urine using semi-preparative HPLC. Typically, 300 µl of urine were injected onto the semi-preparative column, and column effluent was collected. Then, the collected effluent was evaporated to dryness under vacuum, and the residue was dissolved in 1 ml of a water-methanol (87/13) mixture.

Nicotine and derivatives were measured in the SEITA Research Centre, in Orleans, using the Barlow colorimetric method.[20]

Jaffé's picric acid method[21] was used to measure urine creatinine. Expected to observed creatinine ratios were calculated on a basis of 0.210 ± 0.027 mmol creatinine per kg of weight[22] in male subjects.

Subjects

Sixty healthy male volunteers were randomly included in this study. They were 24–55 years old. Thirty volunteers were non-smokers, and 30 volunteers smoked between 15 and 25 cigarettes per day. All smokers inhaled cigarette smoke. Before entering the study, volunteers came to the Grenoble University Hospital where they were weighed, and they were given 21 plastic containers to collect urine. They filled out a questionnaire with the help of a biologist, and then they were given careful written and verbal instructions to collect 24-h urine completely. Volunteers were asked to write in real time what they ate on the day of urine collection. The questionnaires were used to collect data that are summarized in Table I.

Three volunteers were excluded from the study: one smoker because his 24-h creatinine excretion was < 9 mmol, one non-smoker because his HMUra 24-h excretion was more than twice the upper limit of normal HMUra, and one smoker because he declared not to inhale cigarette smoke.

TABLE I Age, weight, height, BMI, creatinine, and creatinine ratio of the two groups of volunteers (mean ± SD)

	Non-smokers ($n = 30$)	Smokers ($n = 30$)
Age (yr)	38.6 ± 9.6	37.9 ± 8.7
Weight (kg)	75.9 ± 13.6	75.0 ± 12.0
Height (m)	175 ± 5.7	175 ± 6.4
BMI	24.4 ± 3.33	24.4 ± 3.36
Creatinine (mmol/24 h)	16.1 ± 2.8	15.0 ± 3.4
Expected to observed creatinine ratio (%)	101 ± 11	95 ± 18

Sample Collection

Volunteers collected urine on the day of their choice. During collection, urine was conserved at +4°C. Volunteers wrote down all they ate and drank during urine collection after each intake. Urine was brought to the laboratory as soon as the 24-h collection was completed. In the laboratory, urine collections were aliquoted and an aliquot was spiked with labelled HMUra. Three aliquots were frozen at −80°C, and two at −20°C. Altered base and nucleoside were measured in urine within one month after collection.

Statistical Analysis

Results were analyzed using PCSM statistical software (Deltasoft, Meylan, France) run on a 386 IBM compatible PC. Distribution normality were tested using the Kolmogorov–Smirnoff test.[23] Normally distributed data were compared using a two-tailed Student's *t*-test. Since 8-oxo-dGuo increase in smokers had been previously described, we used a one-tailed test to compare these data. Differences of $p < 0.05$ were considered statistically significant. Correlation was searched for with forward stepwise multiple regression analysis.[24]

RESULTS

Results concerning ages, weights, heights, BMIs, creatinine, and expected-to-observed creatinine ratios are summarized in Table I. None of these data showed significant variations; however, observed-to-expected creatinine ratio was slightly lower in smokers. This difference is close to significance ($p = 0.059$). No urine collection showed a creatinine ratio lower than 60% or higher than 140%, these limits would show respectively gross incomplete urine collection or urine collection for more than 24-h.[25] Smokers consumed 21.4 ± 3.6 cigarettes per day (mean ± SD).

Plot graphs of 8-oxo-dGuo/24 h vs. 8-oxo-dGuo/Creatinine, 8-oxo-dGuo/24 h vs. 8-oxo-dGuo/kg and 8-oxo-dGuo/kg vs. 8-oxo-dGuo/Creatinine are shown in Figures 1, 2, and 3, respectively.

Results concerning DNA adducts are shown in Table II. Nicotine and derivative excretions were

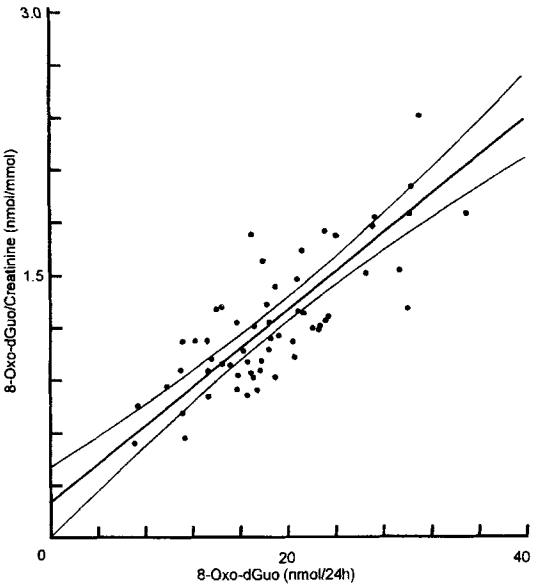

FIGURE 1 Scatter graph and regression line between 8-oxo-dGuo and 8-oxo-dGuo/creatinine ratios. Curves represent 95% confidence limits of the regression line.

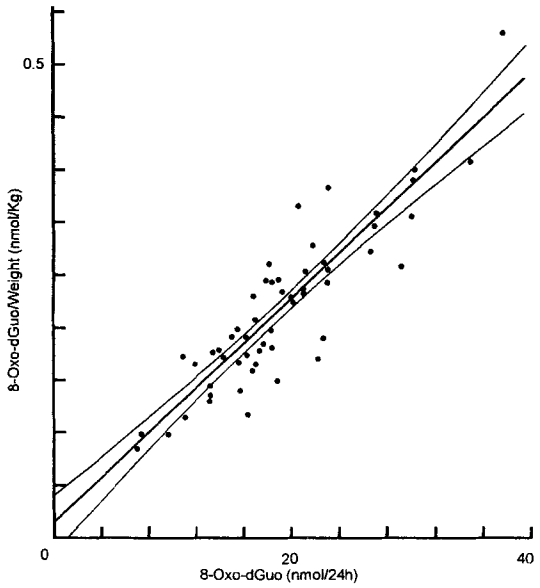

FIGURE 2 Scatter graph and regression line between 8-oxo-dGuo and 8-oxo-dGuo/weight ratios.

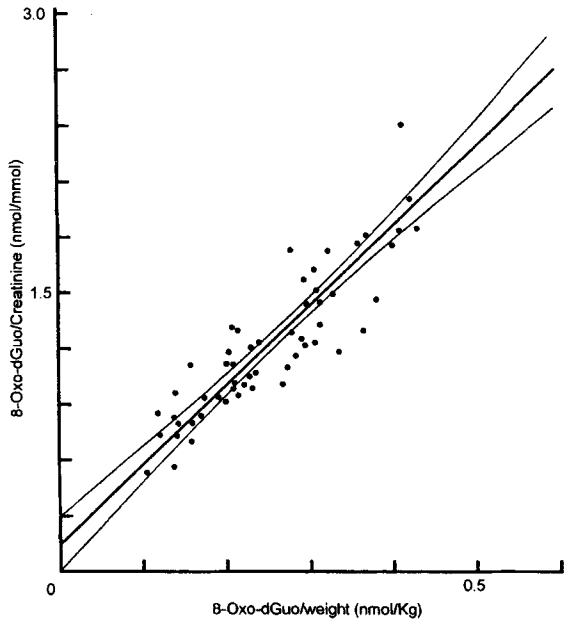

FIGURE 3 Scatter graph and regression line between 8-oxo-dGuo/weight and 8-oxo-dGuo/creatinine ratios.

TABLE II Urine HMUra and 8-oxo-dGuo, and ratios, in smokers and non-smokers (mean ± SD)

	Non-smokers ($n=30$)	Smokers ($n=30$)
Nicotine and derivatives (μmol/24 h)	3.25 ± 0.97	100.5 ± 52.6
Nicotine and derivatives/ Creat. (μmol/mmol)	0.206 ± 0.073	6.86 ± 3.48
HMUra (nmol/24 h)	103 ± 28	100 ± 34
HMUra (nmol/kg)	1.42 ± 0.382	1.35 ± 0.470
HMUra/Creatinine (nmol/mmol)	6.54 ± 2.07	6.70 ± 1.68
8-Oxo-dGuo (nmol/24 h)	18.4 ± 5.7	20.1 ± 7.3
8-Oxo-dGuo (nmol/kg)	0.25 ± 0.08	0.27 ± 0.10
8-Oxo-dGuo/Creatinine (nmol/mmol)	1.16 ± 0.35	1.35 ± 0.50*

* $p < 0.05$ (one-tailed student t-test).

much higher in smokers (mean: 0.206 vs. 6.86, $p < 0.0001$), HMUra/creatinine ratio increased by 2.4% in smokers, but this was not significant ($p = 0.737$). 8-Oxo-dGuo/creatinine ratio increased significantly by 16.3% ($p = 0.042$).

Tables III and IV present the results of forward stepwise multiple regressions for urine HMUra and 8-oxo-dGuo.

TABLE III Forward stepwise multiple linear regression analysis of HMUra

HMUra (nmol/d) dependent variable	r (partial)	β	p
Creatinine (mmol/d)	0.44	0.63	< 0.0001
BMI (kg/m^2)	−0.27	−0.28	0.035
Barlow (μmol/d)	0.26	0.23	0.042

TABLE IV Forward stepwise multiple linear regression analysis of 8-oxo-dGuo

8-Oxo-dGuo (nmol/d) dependent variable	r (partial)	β	p
Creatinine (mmol/d)	0.36	0.50	0.001
Barlow (μmol/d)	0.29	0.29	0.014
BMI (mmol/d)	−0.24	−0.26	0.064

Collected dietary data show that all volunteers consumed typical French foods, which were taken in three meals: breakfast, lunch, and dinner. No volunteer ate any nucleic-acid rich foods (offals, giblets, anchovies, sardines, and herring),[26] which could interfere with DNA adduct excretions.

DISCUSSION

We consider that only the HMUra and 8-oxo-dGuo ratios to creatinine should be taken into account, and that urine must be collected for 24 h. Indeed, creatinine excretion in urine changes during the day, and circadian cycles vary between individuals. Oxidized adducts must be calculated as ratios to creatinine for, at least, two reasons:

- Urine excretion of a compound that is synthesized by body cells depends on body mass. Consequently, interindividual comparisons require corrections for weight and height differences. Creatinine ratios allow for this correction as creatinine is secreted by muscle mass.
- Creatinine excretion corrects either for incomplete or excessive urine collection. A multi-centre lead study in Europe[25] showed

that at least 37.6% of 24-h urine collection was incomplete, and 5.1% of urine collection was collected beyond 24 h. In our study, observed-to-expected creatinine ratios average 101% in non-smokers, but only 95% in smokers although BMIs are equal in the two groups. This discrepancy indicates that smokers did not collect urine as carefully as non-smokers. Therefore, HMUra and 8-oxo-dGuo ratios to creatinine correct the slightly better urine collection in non-smokers. In this study, we verified that there were same number of meat eaters in the two groups, on the day of urine collection.

Para-amino benzoic acid (PABA) had been proposed to control completeness of urine collection;[25] however, it is not satisfactory as it cannot detect collection for more than 24 h. Moreover, volunteers have to take three 80 mg pills of PABA, and some forget, which may bias the study.

Our results show that HMUra excretion is not significantly increased in smokers. As we hypothesized in a previous study,[19] HMUra seems to increase only under acute oxidative stress like adriamycin treatment, and its fast repair by a specific glycosylase allows its detection in urine shortly after the stress. With a higher tobacco consumption, this marker would probably be significantly increased.

Concerning 8-oxo-dGuo, our results are close to those of Tagesson *et al.*,[6] who found a 27.5% increase in smokers. In 1992, Loft *et al.*[7] reported the results of urinary 8-oxo-dGuo from 30 smokers which they compared to 53 non-smokers. They found a 50% increase of urine 8-oxo-dGuo in smokers. Loft's higher results could be the consequence of different methodology and population. Indeed, the authors reported the 8-oxo-dGuo/weight ratio, not 8-oxo-dGuo/creatinine. To insure complete urine collection, they used PABA supplementation. It should also be noted that volunteers from this study were older than ours (mean: 38.25 vs. 51.0 yr). Younger subjects have better antioxidant defence than older ones. Their study included male and female volunteers, while ours included males only. For 8-oxo-dGuo, Loft *et al.* used a sample addition method to measure 8-oxo-dGuo peaks by HPLC while we used a more classical one with a standard curve. Indeed, we injected 300 µl of urine to extract 8-oxo-dGuo by semi-preparative HPLC, and then measured the adduct by coulometry on the total initial amount of 8-oxo-dGuo. Loft's team only injected 70 µl, and column switching allowed them to measure 8-oxo-dGuo on 70% of the initial amount. Our method appears to be more precise (CV = 5.4% vs. 10%) and sensitive (limit of detection: 55 fmol/l vs. 200 pmol/l).

Correlation analysis exhibited dependence of nucleic acid adduct on creatinine, which shows that these adducts are formed in lean body mass. Like Loft *et al.*, we found a negative relationship between BMI and both adducts. The 24-h amounts of HMUra and 8-oxo-dGuo are both correlated to nicotine derivative in urine.

Fraga *et al.* have demonstrated, in rats, that urinary excretion of 8-oxo-Gua is influenced by diet. On the contrary, 8-oxo-dGuo urinary excretion does not depend on dietary conditions.[27] It is therefore not necessary to control the diet when measuring urinary 8-oxo-dGuo. HMUra, like 8-oxo-Gua, is a nucleobase, but the first is a pyrimidine and the second a purine base. Metabolism and absorption specificity of these two compounds are different. Actually, our experience shows that 24-h HMUra excretion is not influenced by dietary conditions, and that it is very stable in the same person from day to day, even under various dietary conditions.[28] Ames and Saul have shown that nucleic acids which are contained in food do not interfere with HMUra excretion. They gave the rats radioactive DNA along with their usual chow;[29] this radioactive DNA contained high levels of tritiated thymine-glycol residues and tritiated 5-(hydroxymethyl)-uracil-residues. Corresponding oxidized residues were recovered as free deoxyribonucleosides in the feces, and none could be found in urine. The French food contains no significant amount

of free bases; this was the case for the meals consumed by volunteers in this study. Bases, nucleotides, and nucleosides which may be present in food can only derive from enzymatic degradation of nucleic acids, provided that the corresponding enzymes exist in the digestive tract. Ames and Saul have shown that dietary DNA cannot interfere with HMUra excretion, which proves that diet does not interfere with urinary HMUra.

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