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Gender Differences in Steady-state Levels of Oxidative Damage to DNA in Healthy Individuals

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Oxidative damage to DNA has often been used as a biomarker for oxidative stress and more specifically for cancer risk. Indeed, the measurement of oxidative damage to DNA, particularly of 8-hydroxyguanine (8OHG) and 8-hydroxy-2′-deoxyguanosine (8OHdG), has been adopted as a method for establishing the effects of antioxidant supplementation towards protection from certain cancers, cardiovascular and neuro-degenerative diseases, both in patients and healthy individuals. However, reported levels of 8OHdG or 8OHG vary considerably, possibly due to the different methodologies used, and only few data are available for the non-smoking and the female population. In this paper, steady-state levels of oxidative damage to DNA measured in a group of 20 males and 19 females are reported. Significant gender differences in levels of modified DNA bases such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy guanine), 8-hydroxy-adenine (8OHA) and 5-hydroxycytosine (5OHC), measured by gas chromatography–mass spectrometry (GC/MS), were observed. The results are discussed in relation to the Vitamin C and iron status of the subjects and to the existing, yet limited, literature data. The role of gender in predisposition to oxidative damage to DNA needs to be addressed in future studies.

Keywords: Oxidative damage to DNA; 8-OH-guanine; Gender; GC/MS

INTRODUCTION

Free radicals and other reactive species are constantly generated *in vivo* and cause oxidative damage to biomolecules, particularly DNA, despite the opposing action of endogenous and dietary antioxidants and the presence of DNA repair enzymes.[1] Experimental and epidemiological evidence has suggested that products resulting from DNA oxidation may be mutagenic and therefore it is increasingly believed that continuous oxidative damage to DNA is a significant contributor to the age-related development of cancer.[2–4] Furthermore, considerable epidemiological evidence has demonstrated a strong correlation between a high intake of fruits and vegetables, which are the principal source of dietary antioxidants, and reduction in cancer risk (reviewed in Refs. [5,6]). As a result, a number of studies have used oxidative damage to DNA as a biomarker for identifying people at risk of developing cancer and for investigating whether dietary antioxidants may be able to lower this risk by lowering oxidative damage to DNA.[7–11]

However, the reported levels of base modifications in DNA, particularly of 8OHdG and 8OHG, vary considerably between different laboratories, not only when different methodologies are employed but also when the same method is used.[12–14] Therefore, it would appear that inter-individual or intra-individual variation can only account in part for the substantial disparity in reported levels and that technical problems, associated with the various methodological approaches, are responsible for the

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discordant results.[7,8] Indeed, accurate measurements are crucial for assessing the extent of oxidative damage to DNA *in vivo* and the biological significance of this damage. Consequently, there is a need for a systematic assessment of steady-state levels of oxidative damage to DNA in large groups of the population, particularly non-smoking and female individuals, as well as males and smokers, before the relevance of changes in oxidative damage to DNA in relation to cancer risk and cancer development and the extent to which antioxidants are able to delay or prevent these processes in humans can be evaluated.

In this paper, we report steady-state levels of nine modified bases, including 8OHG, in DNA derived from white blood cells of healthy non-smoking female and male volunteers. The levels were measured by gas chromatography–mass spectrometry with selective ion monitoring. A method validated to reduce the formation of artefacts and the stable isotope dilution technique for a more accurate quantification of modified bases were used.[15–17]

MATERIALS AND METHODS

Subjects

Forty healthy non-smoking volunteers, 20 males and 20 females, aged between 23 and 46, BMI 23.6 ± 2.4 and 24.3 ± 3.0 kg/m^2, plasma Vitamin C levels 77 ± 12 and 71 ± 9 μmol/l and serum ferritin levels 23 ± 10 and 58 ± 34 μg/l, respectively, were recruited (Table I). After a screening examination on the general state of health by a physician, they provided a fasting blood sample and completed a food frequency questionnaire for the estimation of their men dietary intake. One female withdrew after recruitment for reason unrelated to the study.

Blood Preparation

Blood was collected in heparinised tubes, centrifuged at 2500 *g* for 15 min at 4°C. Following careful removal of the plasma supernatant, the red cell pellet and the buffy coat interface containing the white blood cell layer were stored at −70°C until DNA analysis was carried out.

DNA Extraction, Hydrolysis and Derivatisation

DNA isolation from whole blood with phenol and chloroform:isoamyl alcohol, acid hydrolysis and derivatisation with bis (trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Pierce Chemical, Rockford, IL), acetonitrile and ethanethiol were carried out as reported elsewhere.[15] All chemicals used were of the highest quality available from Sigma-Aldrich Chemical (Poole, Dorset, UK) and BDH Chemical (Gillingham, Dorset, UK).

GC/MS Analysis

Samples were analysed by gas chromatography–mass spectrometry (GC/MS) with selected ion monitoring (SIM), on a Hewlett Packard Model 5890 Series II gas chromatograph (Palo Alto, CA, USA) interfaced to a Hewlett Packard Model 5971 mass selective detector according to the method of Dizdaroglu[16] as modified in Jenner *et al*.[17] Nine modified bases, 8OHG, FAPy guanine, 8-hydroxyadenine (8OHA), 4,6-diamino-5-formamidopyrimidine (FAPy adenine), 5-hydroxymethyluracil (5OH Me uracil), 5-hydroxyhydantoin (5OH hydantoin), 5-hydroxy-5-methylhydantoin (5OH Me hydantoin), 5-hydroxycytosine (5OHC) and *cis*-thymine glycol, were measured. Quantification of modified bases was achieved using stable isotope-labelled standards obtained from Cambridge Isotope Laboratories (Andover, USA) as described by Dizdaroglu.[18] 8-Hydroxyguanine-1,3-$^{15}N_2$-(2-amino-^{15}N)-2-^{13}C, 8-hydroxyadenine-

TABLE I Characteristics of the volunteers

	Mean (range)	
	Females ($n = 19$)	Males ($n = 20$)
Age (y)	29 (23–46)	31 (24–44)
Height (cm)	164 (153–180)	175 (166–192)
Weight (kg)	64 (54–81)	74 (63–98)
BMI (kg/m^2)	24 (19–27)	24 (19–32)
Plasma vitamin C (μM)	77 (54–106)	71 (52–85)
Serum ferritin (μg/l)	23 (6–42)	58 (9–130)
Energy intake (kcal/d)	1706 (1212–2531)	2365 (1194–4138)
Carbohydrate intake (g/d)	219 (128–303)	273 (149–448)
Protein intake (g/d)	76 (45–110)	91 (56–122)
Total fat intake (g/d)	54 (20–91)	68 (19–141)
Vitamin C intake (mg/d)	87 (24–131)	90 (25–170)
Iron intake (mg/d)	11 (5–19)	12 (6–21)

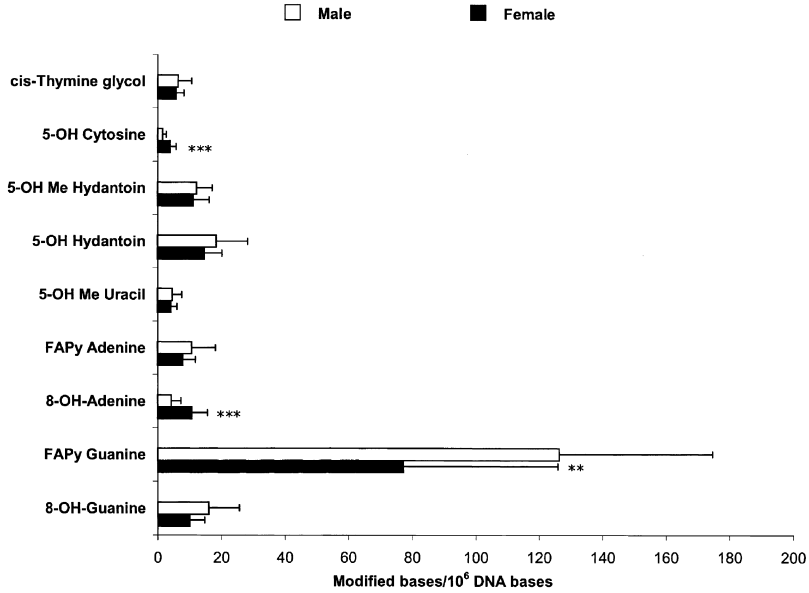

FIGURE 1 Steady-state levels of modified DNA bases in female and male healthy non-smoking subjects. Significant differences between females ($n = 19$) and males ($n = 20$) determined by Mann–Whitney U Test, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$.

1,3,7-$^{15}N_3$-2,8-$^{13}C_2$, 4,6-diamino-5-formamidopyrimidine-1,3-$^{15}N_2$-2-^{13}C-(5-formamido-^{15}N,^{2}H), 2,6-diamino-4-hydroxy-5-formamidopyrimidine-1,3-$^{15}N_2$-(5-amino-^{15}N)-2-^{13}C, 5-hydroxymethyluracil-2,4-$^{13}C_2$-α,α-$^{2}H_2$, 5-hydroxycytosine-1,3-$^{15}N_2$-2-^{13}C and thymine-α,α,α,6-$^{2}H_4$ glycol were dissolved in water to a concentration of *ca.* 1 mg/ml. The exact concentration of the labelled standards was determined by UV spectrophotometry using appropriate extinction coefficients.[18] Because thymine glycol and 5OH hydantoin do not absorb in the UV region, the concentration of these standards was determined by GC/MS. A solution containing a known concentration of unlabelled standard was spiked with labelled standard and analysed by GC/MS. The concentration of labelled compound was then determined by comparison of the peak areas. All labelled standards were added to DNA after isolation, prior to hydrolysis and GS/MS analysis.

Dietary Intake

The mean daily dietary intake of all subjects for the main nutrients was estimated from food intake questionnaires designed for use with DietQ Nutritional Analysis Software Version 3.

Statistical Analysis

Data were analysed by using SPSS 10.0.5 for Windows (SPSS, Chicago, Illinois, USA). Differences in the means between the female and the male group were analysed by using the Mann–Whitney U test and $P \leq 0.05$ was considered significant. Associations between variables were investigated by using multiple linear regression analysis.

RESULTS

The comparison between levels of modified DNA bases of female and male healthy non-smoking individuals is shown in Fig. 1 and in Table II. The results clearly show significant difference in levels of certain modified bases in females compared to males. In fact, FAPy guanine levels in the female group, 77.31 ± 48.66 modified bases/10^6 DNA bases, were significantly lower ($P = 0.003$) than those observed in the male group, 126.28 ± 48.36 modified bases/10^6 DNA bases. On the contrary, 8OHA concentration was higher in females, 10.78 ± 4.93, than in males, 4.31 ± 3.08 modified bases/10^6 DNA bases. Also, levels of 5OHC in the female group, 4.00 ± 1.85 modified bases/10^6 DNA bases, were elevated with respect to those observed for the male counterpart, 1.54 ± 1.23 modified bases/10^6 DNA bases. No significant differences in levels of 8OHG (10.16 ± 4.64 F, 16.02 ± 9.55 M modified bases/10^6 DNA bases), FAPy adenine (8.01 ± 4.00 F, 10.78 ± 7.39 M), 5OH Me uracil (4.31 ± 1.85 F, 4.62 ± 3.08 M), 5OH hydantoin (14.78 ± 5.54 F, 18.48 ± 9.85 M), 5OH Me hydantoin (11.39 ± 4.93 F, 12.32 ± 4.93 M) and *cis*-thymine glycol (5.85 ± 2.46 F, 6.47 ± 4.31 M) were observed between gender. However, a trend for the female subjects to have lower levels of 8OHG, with respect to the males ($P = 0.069$), was observed.

There were no significant gender differences in age, weight, height, BMI, dietary Vitamin C and iron

TABLE II Comparison of steady-state levels of 9 oxidative DNA base damage products in female and male healthy non-smoking volunteers

Modified bases	Mean±SD (modified bases/10^6 DNA bases)	
	Female ($n = 19$)	Male ($n = 20$)
8-OH-Guanine	10.16±4.64	16.02±9.55
FAPy Guanine	77.31±48.66* ($P = 0.003$)	126.28±48.36
8-OH-Adenine	10.78±4.93* ($P = 0.001$)	4.31±3.08
FAPy Adenine	8.01±4.00	10.78±7.39
5-OH Me Uracil	4.31±1.85	4.62±3.08
5-OH Me Hydantoin	11.39±4.93	12.32±4.93
5-OH Hydantoin	14.78±5.54	18.48±9.85
5-OH Cytosine	4.00±1.85* ($P = 0.001$)	1.54±1.23
Cis-Thymine glycol	5.85±2.46	6.47±4.31

*Significantly different from male group as determined by Mann–Whitney *U* test (*P* value in brackets).

intake while the female subjects had lower mean energy, protein and carbohydrate intakes than their male counterparts (Table I). Plasma Vitamin C levels were similar between the two groups and correlated with the mean dietary Vitamin C intake ($r = 0.34$, $P = 0.030$). Despite similar dietary iron intakes, females exhibited a significantly lower iron status ($P = 0.001$); in fact serum ferritin levels were 23 ± 10 and 58 ± 34 μg/l in the female and male group, respectively.

Multiple linear regression analysis showed gender as a significant predictor for 8OHA ($r = 0.63$, $P = 0.001$), FAPy guanine ($r = 0.46$, $P = 0.003$) and 5OHC ($r = 0.57$, $P = 0.001$) while 8OHG was significantly correlated to serum ferritin levels ($r = 0.39$, $P = 0.015$) for which, however, gender was a significant predictor ($r = 0.57$, $P = 0.001$).

DISCUSSION

This report represents one of the few comparisons of steady-state levels of modified DNA bases in a group of normal healthy female and male individuals. Females exhibited significantly lower concentrations of FAPy guanine, than males. However, females had higher levels of 8OHA, a lesion that has been suggested to induce misincorporations in DNA synthesis *in vitro*,[19] and 5OHC, an oxidative product that has exhibited sequence mispairing *in vitro*.[20] They also showed a trend towards lower levels of 8OHG, a putative highly mutagenic lesion, with respect to males.

In general, baseline levels of the majority of modified bases measured in this study were lower than those previously observed by us.[21] From the reanalysis of the data of the study published in Proteggente *et al.*[21] no significant gender differences were found (Table III). However, it is conceivable that in the present study we attained a more accurate quantification of modified bases due to the availability of stable isotope-labelled standards. Furthermore, levels of modified bases can vary over a wide range, being the result of a dynamic equilibrium between rate of damage and rate of repair.

In Table III literature data on basal levels of 8OHdG or 8OHG in non-smoking healthy individuals are summarised. Unfortunately, the availability

TABLE III Comparison between steady-state levels of 8-hydroxyguanine (8OHG) or 8-hydroxy-deoxy-guanosine (8OHdG) found in literature

Study	Subjects	Marker	Technique	Amount (modified bases/10^6 DNA bases)
Loft *et al.*[24]	52 Females	Urinary 8OHdG	HPLC-ED	73.9±33.9 modified bases/kg/24 h*
	31 Males			83.2±30.8
Collins *et al.*[22]	4 Females from France	Lymphocyte	HPLC-ED	5.1†
	4 Males	8OHdG		5.8
	4 Females from Ireland			4.6†‡
	4 Males			9
	4 Females from Spain			3.3†
	4 Males			3.4
Chen *et al.*[23]	20 Females	Leukocyte	HPLC-ED	21.4±3.2¶
	12 Males	8OHdG		25.0±4.7
Lodovici *et al.*[25]	9 Females	Leukocyte	HPLC-ED	16.42±8.65
	22 Males	8OHdG		14.93±10.72
Proteggente *et al.* ([21], reanalysed data)	11 Females	Lymphocyte	GC/MS	17.86±9.86*
	9 Males	8OHG		18.48±8.62
This study	19 Females	Lymphocyte	GC/MS	10.16±4.64
	20 Males	8OHG		16.02±9.55

*Value obtained by using the following conversion factor: 308 8OHdG/10^6 DNA bases=1 nmol 8OHdG/mg DNA[8].
†Pooled samples.
‡Significantly different from male group by ANOVA ($P = 0.004$)[22].
¶Significantly different from male group by Student *t*-Test ($P < 0.01$)[23].

of data on oxidative damage to DNA, which are differentiable by gender and methodologically comparable, is limited because the majority of studies have measured only 8OHdG in male and/or smoker or mixed gender groups. Collins *et al.*[22] have found that levels of 8OHdG from lymphocytes of healthy non-smoking women from Ireland were lower than those observed in men. However, in the same study no difference between levels of 8OHdG between groups of non-smoking men and women either from France or from Spain was observed. Also, Chen *et al.*[23] observed that levels of 8OHdG in non-smoking women were lower than those measured in men. Furthermore, Loft *et al.*[24] have measured the urinary excretion of 8OHdG in healthy subjects and found that gender is one of the important predictors of the 8OHdG excretion. In fact, according to multiple regression analysis men excreted 29% more 8OHdG than women (240 ± 106 and 271 ± 96 pmol/kg/24 h, respectively), although this difference was not significant. However, Lodovici *et al.*[25] found no difference between genders in healthy non-smoking men and women.

Therefore, some observations would suggest a potential gender determinant in the susceptibility to oxidative damage to DNA but others would indicate the opposite. However, it would appear that levels of 8OHdG and 8OHG measured in these studies by using different methods, high performance liquid chromatography with electrochemical detection (HPLC-ED) and GC/MS, are generally similar. Therefore, one might deduce that methodological differences cannot account for the disparity of the results and that instead an insufficient number of individuals in the studies and the influence of dietary, lifestyle and environmental factors and iron status are likely to be the cause. In conclusion, it remains unclear at this stage whether gender differences in oxidative damage to DNA exist but undoubtedly the experimental data are so limited that further investigations on a larger scale and taking into account all the confounding factors are necessary before conclusions can be drawn.

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