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Iron Supplementation and Oxidative Damage to DNA in Healthy Individuals with High Plasma Ascorbate

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Previously, we have investigated the potential for a pro-oxidant interaction of iron and ascorbate *in vivo* in iron and ascorbate cosupplementation or ascorbate supplementation studies. In this study, for the first time, the effects of iron supplementation on oxidative damage to DNA in healthy individuals with plasma ascorbate levels at the upper end of the normal range were examined. Forty female and male volunteers (mean plasma ascorbate ≈70 μmol/L) were supplemented with a daily dose of syrup (ferrous glycine sulphate equivalent to 12.5 mg iron) for 6 weeks. Serum ferritin, transferrin bound iron, % saturation of transferrin and plasma ascorbate were assessed and the mean dietary intakes of all subjects were estimated through food frequency questionnaires. Oxidative damage to DNA bases from white blood cells was measured by gas chromatography/mass spectrometry with selected-ion monitoring (GC/MS-SIM), using isotope-labelled standards for quantification. Iron supplementation did not affect any of the iron status parameters. There were also no detrimental effects, over the period under investigation, in terms of oxidative damage to DNA. However, the effects of larger doses or of longer supplementation periods should also be investigated.

Key Words: **iron; ascorbate; supplementation; oxidative damage; DNA; gender.**

There is abundant epidemiological evidence that a diet rich in fruit and vegetables can protect against cancer (1, 2). In particular, numerous epidemiological studies have reported an association between vitamin C intake or plasma levels and protection against some cancers (3). Although the extent of the link between DNA oxidation and cancer is debated (4, 5), the potent free radical scavenging abilities of vitamin C, which could prevent oxidative DNA damage, have been advocated as a potential anti-carcinogenic mechanism. However, the benefits and the effects of vitamin C supplementation on oxidative DNA damage or cancer prevention remain unclear (6). Similarly, interactions due to complementary vitamins and minerals have yet to be extensively studied. Particularly, the interaction between vitamin C and iron has raised some concern due to the well known ability of ascorbate to reduce Fe^{3+} to Fe^{2+} and consequently to promote the formation of hydroxyl radicals *in vitro* if H_2O_2 is available (7–10).

In previous studies we have investigated the effects of iron and ascorbate co-supplementation on oxidative DNA *in vivo* and obtained little clear-cut evidence for a pro-oxidant effect (11, 12). However, the effects of iron supplementation on oxidative DNA damage have never been investigated. The consumption of dietary supplements which contain at least the RDA of iron (14 mg/d), has become widespread within the general public (13–15). Yet, insufficient information is available on the potential side effects of iron supplementation or benefits in normal individuals. In fact, although iron is an essential element in the human body for the synthesis of various enzymes and proteins involved in the cellular respiration chain and oxygen transport, it is also a powerful promoter of free-radical reactions *in vitro* (16). Furthermore, elevated body iron stores might be associated with an increase in cancer risk and coronary heart disease (17, 18).

The present study has investigated, for the first time, whether supplementation with a level of iron widely consumed by the general public can modulate DNA damage in healthy individuals with dietary ascorbate levels at the upper end of the normal range.

MATERIALS AND METHODS

Study protocol. Ethical permission for this study was obtained from the Guy's Research Ethics Committee (No. 00/01/26). Forty healthy non-smoking volunteers, 20 males and 20 females, aged between 23 and 46 years, BMI 23.64 ± 2.45 Kg/m^2, were recruited

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TABLE 1

Characteristics of the Volunteers

	Mean (range)	
	F (n = 18)	M (n = 19)
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 ascorbate, μmol/L	77 (54–106)	71 (52–85)
Ferritin, μg/L	23 (6–42)	58 (9–130)
Transferrin Bound Iron (TBI), μmol/L	12 (4–20)	18 (6–29)
Saturation of Transferrin (ST), %	25 (7–41)	33 (13–56)

(Table 1). Exclusion criteria were iron deficiency or anaemia, smoking, high alcohol intake (men >25 units/wk and women >20 units/wk), pregnancy, use of oral contraceptives, consumption of vitamin and mineral supplements or drugs. Subjects underwent medical examination by a physician and preliminary blood and urine tests were carried out. The volunteers were supplemented for 6 weeks with proprietary brand syrup containing 12.5 mg/d iron as ferrous glycine sulphate (Plesmet, Link Pharmaceuticals Ltd., Horsham, UK). Fasting venous blood samples were collected prior to the start of supplementation and again after 6 weeks. Plasma ascorbate, serum iron and white blood cells DNA base damage were measured on both occasions. Thirty-nine volunteers completed the study (one female withdrew due to reasons unrelated to the study). Subject compliance was determined by measuring the volume of syrup returned at the termination of the study and was estimated to be over 90%. No adverse events related to the treatment were observed. Two subjects were excluded from the results as extreme outliers.

Plasma ascorbate. Sample preparation and measurement according to a fluorimetric method were carried out as reported elsewhere (12).

Serum iron. Serum ferritin was measured spectrophotometrically on a SPECTRAmax 190 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) by using an Enzyme Immunoassay Kit (Spectroferritin, Ramco Laboratories Inc., Mt. Vernon, Houston, TX) based on a published method (19). Transferrin bound iron (TBI) and % saturation of transferrin (ST) were determined by using a Serum Iron/UIBC Assay Kit (Randox Laboratories Ltd., Ardmore, UK) as previously described (12).

Oxidative damage to DNA. DNA isolation from whole blood, hydrolysis and derivatisation were carried out as published elsewhere (20). Samples were analysed by gas chromatography-mass spectrometry with selected-ion monitoring (GC/MS-SIM), on a Hewlett Packard Model 5890 Series II gas chromatograph (Palo Alto, CA) interfaced to a Hewlett Packard Model 5971 mass selective detector. The conditions of analysis were as previously reported (21). Quantification of base products was achieved by using stable isotope labelled standards (22). 8-Hydroxyguanine-1,3-$^{15}N_2$-(2-amino-^{15}N)-2-^{13}C, 8-hydroxyadenine-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,^2H), 2,6-diamino-hydroxy-5-formamido-pyrimidine-1,3-$^{15}N_2$-(5-amino-^{15}N)-2-^{13}C, 5-hydroxymethyl-uracil-2,4-$^{13}C_2$-α,α-$^2H_2$, 5-hydroxy-cytosine-1,3-$^{15}N_2$-2-^{13}C; thymine-α,α,α,6-$^2H_4$ glycol were purchased from Cambridge Isotope Laboratories (Andover, USA) and dissolved in water to a concentration of ca. 1 mg/ml. The exact concentrations of the standard solutions were determined by UV spectrophotometry using appropriate extinction coefficients, prior to their addition to DNA after isolation (22).

Dietary intake. The mean dietary intakes of the main nutrients were estimated from food frequency questionnaires designed for use with DietQ Nutritional Analysis Software Version 3 (Tunuviel Software, Warrington, UK).

Statistical analysis. Statistical advice on the number of subjects to be used in the study was obtained prior to starting. On the basis of the observations of a previous study (11), 20 individuals in each group were established as a sufficient number to yield a power of at least 90%. Statistical analysis was carried out with SPSS 9.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Normality of distribution was assessed using Kolmogorov-Smirnov Goodness of Fit Test. One-way ANOVA and Bonferroni Multiple Comparisons *post hoc* test were used to compare differences in the mean between and within groups. Correlations between variables were determined by Pearson's correlation coefficient. In all cases, $P \leq 0.05$ was considered significant.

RESULTS

Plasma ascorbate concentrations were similar, in the female and the male groups, prior to iron supplementation (77 ± 3 and 72 ± 2 μmol/L, respectively) and were unchanged after 6 weeks of supplementation (74 ± 3 to 72 ± 4 μmol/L, respectively). Plasma ascorbate concentrations measured at baseline were as expected from the estimated daily dietary intake of vitamin C (Table 2).

Serum concentrations of ferritin were different between females and males, both at the start of the study (23 ± 2 and 58 ± 8 μg/L, respectively) and at the end of the supplementation period (26 ± 3 and 70 ± 9 μg/L, respectively). Typically, serum ferritin concentrations in females were significantly lower than in males, both pre- (P = 0.001) and post-supplementation (P = 0.001). No changes due to supplementation were observed (Fig. 1). However, there was a wide range of response with a trend to increase in the male group, as highlighted in Fig. 2 where the individual responses of (A) serum ferritin and (B) TBI to iron supplementation are presented. Serum ferritin concentrations were correlated with TBI at baseline (r = 0.34, P = 0.04) and at 6 weeks (r = 0.38, P = 0.02). Also, there were no significant differences in TBI and %ST following supplementation (Fig. 1). In fact, baseline concentrations

TABLE 2

Estimated Mean Daily Dietary Intake of Main Nutrients of the Volunteers

	Mean (range)	
Intake	F (n = 18)	M (n = 19)
Energy, MJ	7160 (5093–10637)	9848 (5020–17204)
Protein, g	75 (45–110)	90 (56–122)
Total fat, g	54 (20–91)	69 (19–141)
Sugars, g	218 (128–303)	271 (149–448)
Fiber, g	19 (12–27)	21 (13–35)
Vitamin C, mg	87 (24–131)	91 (25–170)
Iron, mg	11 (5–19)	12 (6–20)

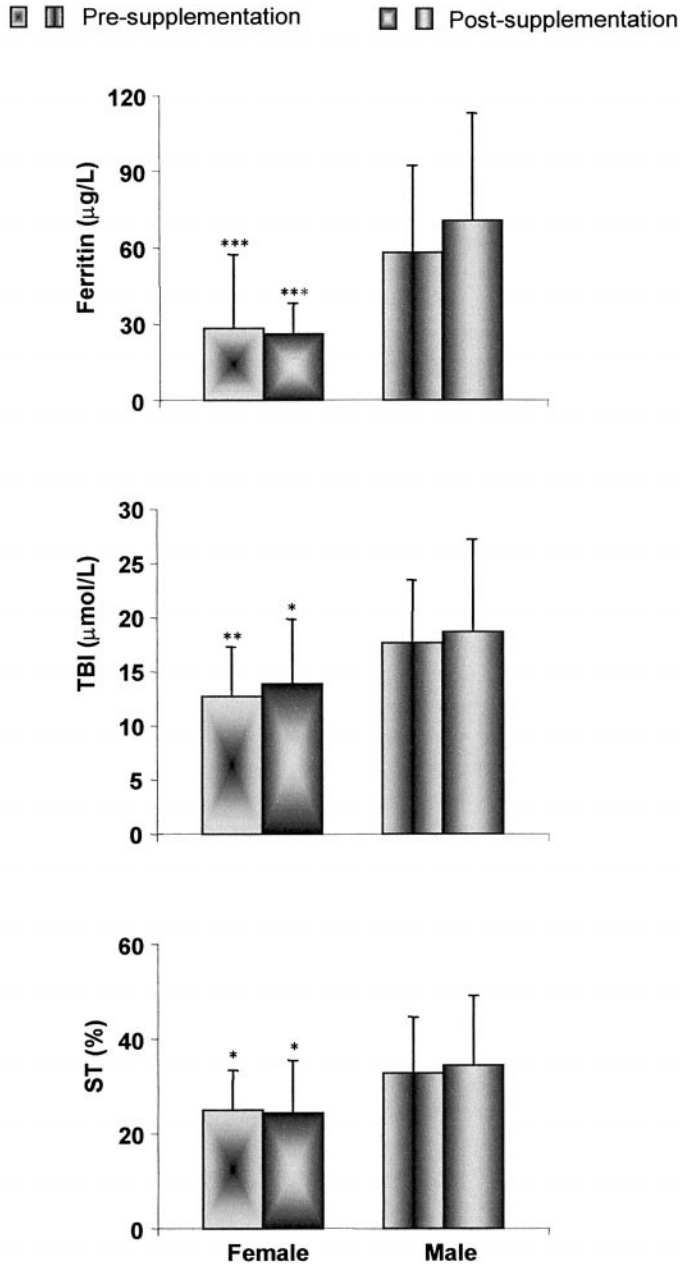

FIG. 1. Mean concentrations of serum Ferritin (μg/L), Transferrin Bound Iron (TBI, μmol/L) and Saturation of Transferrin (ST, %) prior to supplementation and after 6 weeks of supplementation with 12.5 mg/d iron, in female (n = 18) and male (n = 19) groups. Statistically significant difference from male group at same time point by One-Way ANOVA with Bonferroni Multiple Comparisons *post hoc* test, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

of TBI were 12 ± 5 μmol/L in the female group and 18 ± 6 μmol/l in the male group and, after supplementation, they were 13 ± 6 and 18 ± 8 μmol/L, respectively.

Total measured damage to DNA base, calculated as the sum of all the products measured, was significantly lower in the female (F) than in the male (M) group, both prior to start (F 146.61 ± 65.60 modified bases/10^6 DNA bases and M 200.82 ± 62.83 modified bases/10^6 DNA bases, respectively) and after supplementation (F 140.45 ± 68.07 modified bases/10^6 DNA bases and M 207.60 ± 51.74 modified bases/10^6 DNA bases, respectively), although no effect of supplementation was observed. Interestingly, TBI and total DNA damage levels where correlated prior to supplementation (r = 0.48, P = 0.002). In addition, large gender differences were observed between levels of specific modified base products, 8-hydroxy-guanine, FAPy guanine, 8-hydroxy-adenine, FAPy adenine, 5-hydroxy-hydantoin and 5-hydroxy-cytosine, but they were unaffected by iron supplementation (Table 3).

DISCUSSION

The results of this study indicate that supplementing individuals with 12.5 mg/d iron for six weeks has no adverse effect on oxidative damage to DNA, even in the presence of high plasma ascorbate concentrations. However, the dose used might have been too low or the length of supplementation too short to produce significant changes in serum iron status and consequently in DNA base damage.

It has been shown that longer periods of supplementation and/or higher doses of iron may be effective in increasing serum iron concentrations. For example, it has been suggested that iron supplementation with 60 mg/d for 3 months or 60 mg once weekly for the same length of time, progressively improves iron status in healthy menstruating women (23). Therefore, it would appear that doses of iron at least 4-fold higher than the RDA could produce an effect on iron status. However, for this study we have chosen to use an iron oral supplement that in a single daily dose would provide an amount of iron close to the RDA. This is for two reasons: firstly, the RDA is the dose of iron most commonly used by the general public, although in combination with vitamins and other minerals. In fact, a supplement containing only the RDA of iron was not commercially available at the time of this study but we were able to obtain iron syrup which, administered daily at the dose of 2.5 ml, would provide 12.5 mg. Secondly, side effects such as diarrhoea and/or constipation, nausea and flatulence frequently manifest at doses higher than the RDA. Furthermore, the benefit of iron supplementation, especially at high doses, has been questioned in view of the fact that excess unabsorbed iron could be implicated in the incidence of colon cancer. For instance, it has been proposed that a fraction of iron, which remains unabsorbed in the small intestine, may enter the colon and participate in a Fenton-type reaction, increasing the formation of free radicals at the mucosal surface (24). Also, reactive species or iron may enter the colonocytes and increase the risk of DNA damage. Moreover, changes in crypt cell proliferation in rat large intestine associated with

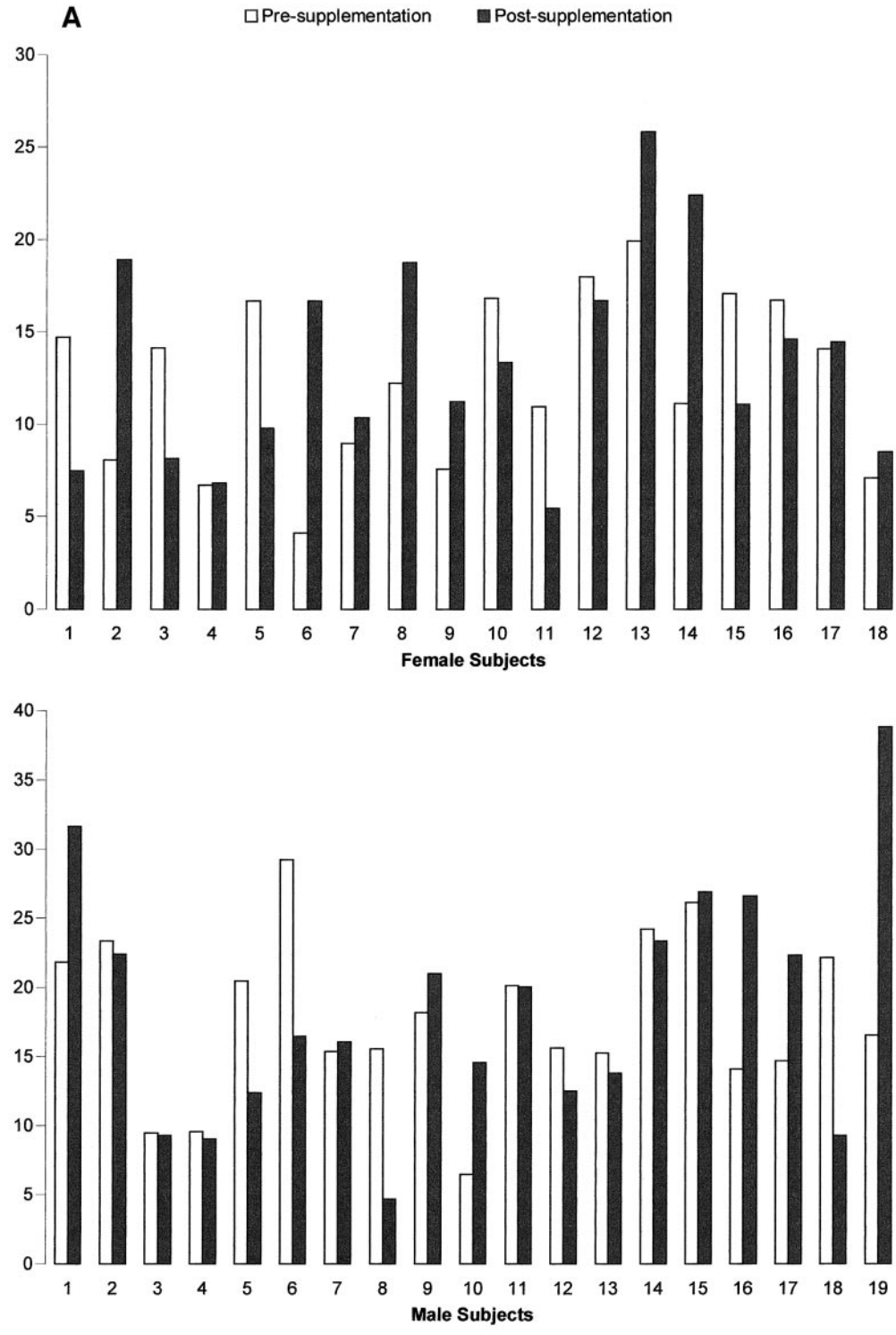

FIG. 2. Individual responses of A) TBI (Transferrin Bound Iron, μmol/L) and B) serum Ferritin concentrations (μg/L) to supplementation with 12.5 mg/d iron for 6 weeks in female (n = 18) and male (n = 19) groups. Mean concentrations obtained of two measurements, in duplicate, for each sample are reported.

increase in the concentration of available iron following to dietary supplementation have been showed (25). The excess iron could be available for free radical reactions to take place in the intestinal lumen. It has also been reported that oral ferrous sulphate supplementation with a dose higher than the RDA (19 mg elemental iron/d) was able to produce an increase in the free radical-generating capacity of faeces in healthy volun-

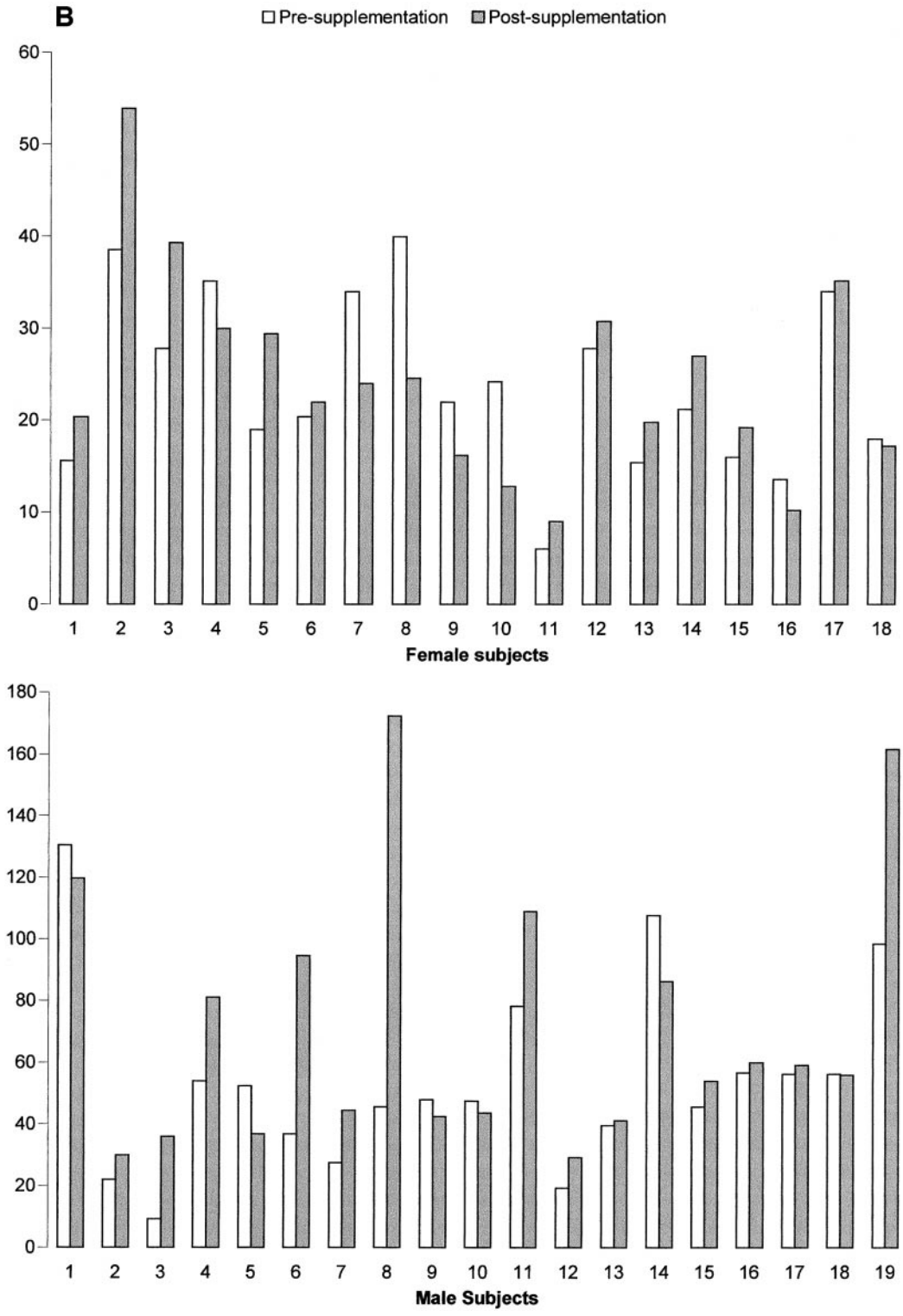

FIG. 2—*Continued*

teers. Hence, it was suggested that unabsorbed iron might increase free radical production in the colon to a level that could cause mucosal cell damage or increased production of carcinogens (26).

Baseline levels of the majority of oxidised base products measured in this study were lower than those observed by us in previous supplementation studies in which the same DNA isolation, hydrolysis, derivatisa-

TABLE 3

Oxidative Damage to DNA Bases Prior to Iron Supplementation and after 6 Weeks of Supplementation

Modified bases/10^6 (DNA bases)	Mean ± SD			
	F (n = 18)		M (n = 19)	
	0 week	6 week	0 week	6 week
8-Hydroxy-guanine	10.16 ± 4.64[a] (P = 0.020)	9.24 ± 3.70[a] (P = 0.006)	16.02 ± 9.55	16.63 ± 10.78
FAPy Guanine	77.31 ± 48.66[a] (P = 0.003)	77.00 ± 53.90[a] (P = 0.003)	126.28 ± 48.36	129.05 ± 48.36
8-Hydroxy-adenine	10.78 ± 4.93[a] (P = 0.001)	10.16 ± 5.24[a] (P = 0.023)	4.31 ± 3.08	4.31 ± 2.16
FAPy Adenine	8.01 ± 4.00	7.08 ± 4.00[a] (P = 0.026)	10.78 ± 7.39	11.09 ± 6.47
5-OH Me Uracil	4.31 ± 1.85	4.00 ± 1.54	4.62 ± 3.08	4.93 ± 3.08
5-OH Me Hydantoin	11.39 ± 4.93	10.78 ± 5.24	12.32 ± 4.93	13.24 ± 4.93
5-OH Hydantoin	14.78 ± 5.54	13.86 ± 5.24[a] (P = 0.021)	18.48 ± 9.85	19.10 ± 8.01
5-OH Cytosine	4.00 ± 1.85[a] (P = 0.001)	3.39 ± 1.54	1.54 ± 1.23	2.46 ± 3.08
Thymine glycol	5.85 ± 2.46	5.24 ± 1.54	6.47 ± 4.31	6.47 ± 3.70
Total measured damage	146.61 ± 65.60[a] (P = 0.012)	140.45 ± 68.07[a] (P = 0.002)	200.82 ± 62.83	207.60 ± 51.74

[a] Significantly different from male group at the same time point by One-Way ANOVA with Bonferroni Multiple Comparisons *post hoc* test.

tion and GC/MS analysis procedures had been adopted, but in that case quantification of DNA damage was not achieved by using stable isotope labeled standards as applied here (11, 12). Thus, although levels of modified bases can vary as a result of the dynamic equilibrium between rates of oxidative damage to DNA and rates of repair of this damage, it is possible that in the present study a more accurate quantification of DNA damage was attained due to the use of isotopically labeled standards (22). For example, in this study, mean baseline levels of 8-hydroxy-guanine were 10.16 ± 4.64 and 16.02 ± 9.55 modified bases/10^6 DNA bases in females and males, respectively. These values are circa 1- to 1.8-fold lower than those reported in Proteggente *et al.* (12) (18.48 ± 9.24 modified bases/10^6 DNA bases in healthy male and female volunteers) and 4.5- to 7-fold lower than those published by Rehman *et al.* (11) (70.84 ± 33.88 modified bases/10^6 DNA bases in healthy male and female individuals). Furthermore, the levels reported here are in the range of those indicated by others using HPLC with electrochemical detection methods (27–28). In fact, Lodovici *et al.* (27) reported 15.3 ± 1.8 8-hydroxy-deoxyguanosine/10^6 DNA bases in healthy female and male non-smokers and Chen *et al.* (28) indicated 21.4 ± 3.2 and 25.0 ± 4.7 8-hydroxy-deoxyguanosine/10^6 DNA bases in healthy females and males, respectively.

Interestingly, significant differences in total oxidative damage to DNA were observed between gender, with women generally showing lower values both pre- and post-supplementation, suggesting a potential gender determinant in the susceptibility to oxidative DNA damage which could be linked to iron status. There is very little data on gender differences and DNA damage in humans; however the current literature would suggest that gender might have an influence on susceptibility to DNA damage (29). However, further investigation would be required in order to substantiate this possibility.

Overall, our findings suggest that supplementation with a dose of iron almost equivalent to the RDA is sufficient to maintain a normal iron status, in healthy individuals with high ascorbate intake from the diet, without inducing disturbing side effects or raising health concerns relative to the potential pro-oxidant nature of ascorbate in the presence of iron, under the conditions of this study. Larger doses, which might entail a higher incidence of side effects and might pose other health risks, should only be used therapeutically in the case of a diagnosed deficiency status or in specific physiological conditions.

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